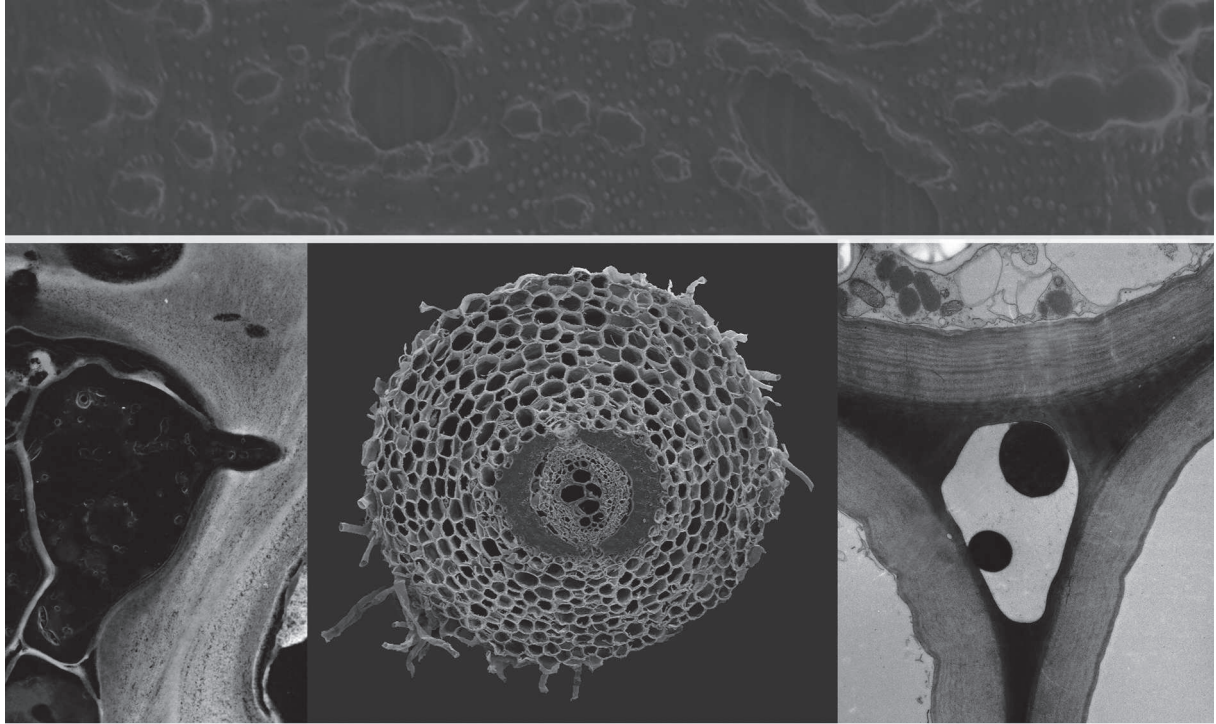
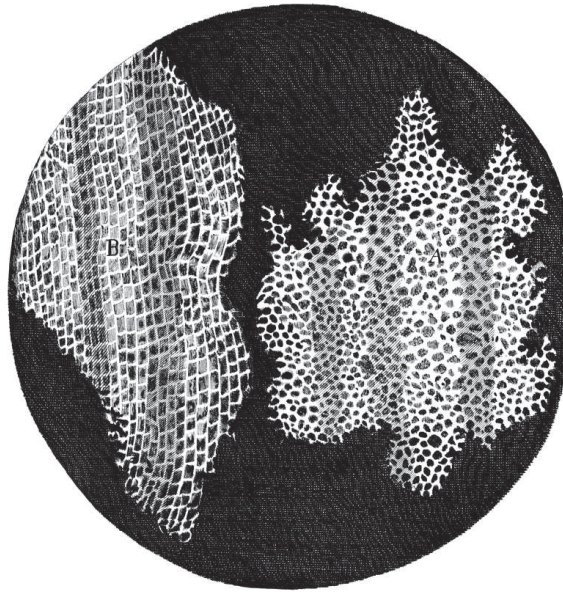




Fern histology: A focus on cell walls and Aspleniaceae

Olivier Leroux

Fig:1.



. . . I could exceedingly plainly perceive it to be all perforated and porous, much like a Honey-comb, but that the pores of it were not regular . . . these pores, or cells, . . . were indeed the first microscopical pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this . . .

Drawing of dried cork tissue¹

Robert Hooke coined the term 'cell' for describing units in plant tissues.

¹ Robert Hooke (1665) *Micrographia: or, some physiological descriptions of minute bodies made by magnifying glasses*. J. Martyn and J. Allestry, London.

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**FERN HISTOLOGY:
A FOCUS ON CELL WALLS AND
ASPLENIACEAE**

**VAREN HISTOLOGIE:
EEN FOCUS OP CELWANDEN EN
ASPLENIACEAE**

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Part 1

Introduction & technical papers

1

General introduction

1.1 HISTORY OF (FERN) ANATOMY: A BRIEF SUMMARY

The invention and subsequent improvement of the microscope was the starting point for plant anatomical studies. Hooke (1665), Grew (1671, 1682), Malpighi (1675), and van Leeuwenhoek (1685) were pioneers, studying the anatomy of stems, roots, flowers and seeds of angiosperms. The earliest studies on fern anatomy were undertaken by Bischoff (1828) on the vascular bundles and carinal canals in *Equisetum* rhizomes, and Brongniart (1828-1838) on the vascular bundles in petioles and stems of ferns and lycophytes. However, in the first half of the 19th century only few studies or surveys on plant anatomy were published.

In the mid 1800s, there was a renewed interest in all parts of botanical research, including anatomy, physiology, and morphology. Research on plant development increased, mainly by von Mohl (1851), who restored the study of anatomy summarizing earlier work in his book "Grundzüge der Anatomie und Physiologie der vegetabilischen Zelle", and Nägeli (1845, 1863) who founded the theory of cell formation. Again, the success of both researchers was due to the continued improvement of microscopical techniques. Attention was also no longer restricted to seed plants but was extended to mosses, lycophytes and ferns. Especially in the second half of the 19th century, anatomical studies on ferns and lycophytes increased and many studies on different aspects of their structure and anatomy were published. Some of the more important works are discussed in detail by von Sachs (1890) and Ogura (1972). The studies of many researchers, including those working on fern anatomy, improved the understanding of general plant anatomy and development, and formed the basis for influential encyclopedia such as "Lehrbuch der Botanik" by von Sachs (1868), "Vergleichende Anatomie der Vegetationsorgane der Phanerogamen und Farne" by de Bary (1877), "Traité de botanique" by van Tieghem (1886-1888), and "Histologische Beiträge" by Strasburger

(1888). Since de Bary (1877) many anatomical investigations of ferns and lycophytes were carried out in greater detail, often with a focus on systematic anatomy, and more precisely on the types of vascular bundles or stele.

By the end of the 19th century the systematic anatomy had also been undertaken in fossil specimens which created more interest in phylogenetic relationships among the major groups of land plants. Soon the anatomical investigation of ferns and lycophytes were undertaken from a comparative, systematic, phylogenetic, or developmental point of view, resulting in an increasing amount of publications. These studies, too numerous to summarize here, are reviewed by Schoute (1938), Bower (1963) and Ogura (1972).

Twentieth century science grew out of the solid foundations laid by the detailed experimental observations of the 19th century. Botany was soon studied at all levels of plant organization from molecules to populations. Botanical advance was also closely associated with technical advances in physics and chemistry. Transmission and scanning electron microscopy opened new possibilities for detailed studies at higher resolutions taking anatomy into the world of “ultrastructure” in the mid 1900s. In the last decade of the 20th century, DNA technology saw immense progress, resulting in the use of DNA sequence data in various botanical disciplines including molecular biology and phylogenetic systematics. The most recent developments in light microscopy largely centre on the use of fluorescence microscopy (epifluorescent and laser scanning confocal microscopes) to carry out fluorescent labelling experiments. These advances led to an enormous amount of publications and only relevant literature will be cited below. However, despite these advances, both in knowledge and technology, the development, (ultra)structure and cell wall composition of fern tissues has not received much attention, mainly because of the traditionally angiosperm-centred approach in many botanical disciplines.

1.2 FERNS AND LYCOPHYTES

Ferns and lycophytes are spore-producing or “seed-free” vascular plants, and because of this shared feature their members have been grouped historically as “pteridophytes” or “ferns and fern allies”, which are paraphyletic assemblages (Schuettpeitz *et al.*, 2008; Pryer *et al.*, 2004). Over the past two decades, a large number of studies have been carried out attempting to reconstruct various parts of the (land) plant phylogeny (e.g., Raubeson and Jansen, 1992; Chase *et al.*, 1993; Garbary *et al.*, 1993; Manhart, 1994; Mishler *et al.*, 1994; Nixon *et al.*, 1994; Rothwell and Serbet, 1994; Hasebe *et al.*, 1995; Pryer *et al.*, 1995, 2001, 2004; Kranz and Huss, 1996; Kenrick and Crane, 1997; Doyle, 1998; Wolf *et al.*, 1998; Soltis

et al., 2002; Chaw *et al.*, 2000; Nickrent *et al.*, 2000; Qui *et al.*, 2006, 2007; Schuettpelz *et al.*, 2008). A current view of the relationships between the major groups of land plants is shown in figure 1.1. It is now commonly agreed that within the tracheophytes a fundamental split occurred in the mid-Devonian, separating a group including the modern lycophytes from a group containing all ferns and seed plants, the euphyllophytes (Pryer *et al.*, 2004). The extant lycophytes possess lycophylls (= microphylls), leaves with an intercalary meristem, and comprise three main clades: homosporous Lycopodiales (clubmosses), heterosporous Isoetales (quillworts), and heterosporous Selaginellales (spikemosses). Euphyllophytes are characterized by euphylls (= macrophylls, leaves with marginal or apical meristems and leaf gaps), lateral branches that terminate in 'sporangia', and a distinctively lobed primary xylem strand (Stein, 1993; Kenrick and Crane, 1997). Extant euphyllophytes comprise two major clades: the spermatophytes (seed plants), with over 260000 species (Thorne, 2002), and the ferns (monilophytes, *sensu* Pryer *et al.*, 2004), with approximately 9000 species (Smith *et al.*, 2006). The latter group is, in most cases, characterized by lateral root origin in the endodermis, mesarch protoxylem in shoots, a pseudoendospore, plasmodial tapetum, and sperm cells with 30–1000 flagellae (Renzaglia *et al.*, 2000). Extant members belong to five major lineages: whisk ferns (Psilotales), ophioglossoid ferns (Ophioglossales), horsetails (Equisetopsida), marattioid ferns (Marattiales), and leptosporangiate ferns (Polypodiales). As relationships among the horsetail, marattioid, and leptosporangiate fern lineages remain unclear (Pryer *et al.*, 2001) they are shown as a polytomy in figure 1.1. The majority of extant ferns belong to the leptosporangiate clade which has diverged in an environment dominated by angiosperms (Schneider *et al.*, 2004b), and therefore faced selective pressures similar to those experienced by seed plants (Thomas and Spicer, 1987; Stewart and Rothwell, 1993). For a modern review on the classification of ferns we refer to Smith *et al.* (2006) and to Schuettpelz *et al.* (2008).

As all other vascular plants, ferns are characterised by an alternation of generations, with a diploid sporophytic and a haploid gametophytic phase. In ferns and lycophytes, both phases are free-living. The gametophyte is a green autotrophic structure attached to substrates by rhizoids and bearing antheridia and archegonia. The sporophyte consists of a rhizome or stem with fronds (leaves) and adventitious roots. Most fern fronds consist of a petiole (stipe) and a lamina with rachis (central axis). They have a circinnate vernation by which young fronds are curled up in a crozier (fiddle head). Rhizomes and fronds are often densely covered with scales (paleae), glands, and/or hairs. The lamina may be simple to finely divided. Primary divisions (pinnae)

may be further divided into pinnules. Sporangia are aggregated into sori, which are, in some genera, protected by indusia.

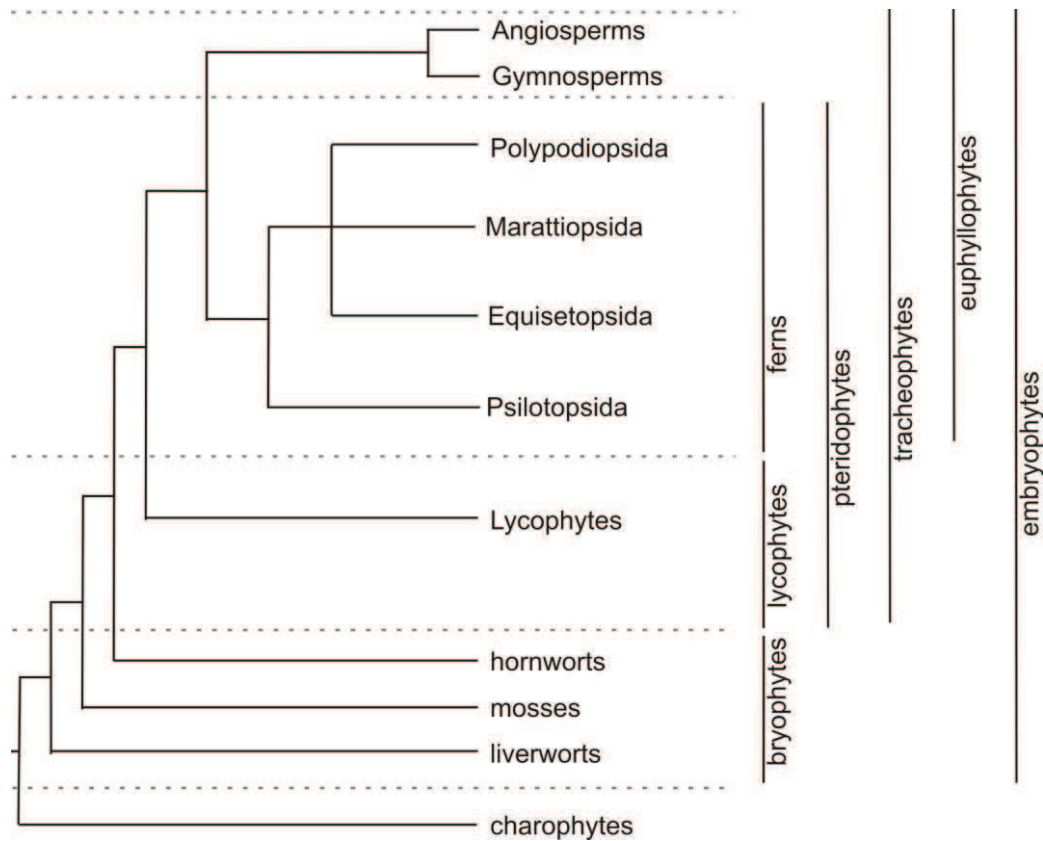


Figure 1.1: Schematic diagram showing phylogenetic relationships among different major groups of (land) plants. Adapted from Smith *et al.* (2006) and Qiu *et al.* (2007).

1.3 ASPLENIACEAE

The family Aspleniaceae (Fig. 1.2) is one of the most species-rich families among leptosporangiate ferns with over 720 species of terrestrial, epilithic or epiphytic ferns distributed all over the world in (sub)humid tropics, south-temperate, and even extending into Arctic and arid areas (Kramer and Viane, 1990). The majority of the species belong to the genus *Asplenium* (Linnaeus, 1753) and of the members of the once recognized segregate genera *Camptosorus* (Link, 1833), *Ceterach* (Willd, 1810), *Diellia* (Brackenridge, 1854), *Loxoscapha* (Moore, 1853), *Pleurosorus* (Fée, 1852), and *Phyllitis* (Hill, 1756) – in most cases based on single, easily observed characters of little taxonomic

weight (Kramer and Viane, 1990) – clearly nest within *Asplenium* s.l. (Murakami *et al.*, 1999; Gastony and Johnson, 2001; Pinter *et al.*, 2002; Van den heede *et al.*, 2003, Schneider *et al.*, 2004a; Bellefroid *et al.*, 2010). Other putative segregate genera such as *Antigramma* (Presl, 1836), *Holodictyum* (Maxon, 1908), *Schaffneria* (Fée, 1855), and *Sinephropteris* (Mickel, 1976) have not been sampled yet for molecular analysis, but it is expected that these also nest in *Asplenium* (Kramer & Viane, 1990; Smith *et al.*, 2006). *Hymenasplenium*, on the other hand, appears to represent the sister clade to the rest of the species in the family. This is supported by molecular (Murakami *et al.*, 1999; Schneider *et al.*, 2004), anatomical (Murakami, 1995; Schneider, 1997) and cytological data (Murakami, 1995).

The rhizomes of *Asplenium* species are creeping, ascending or suberect, dictyostelic and covered with clathrate scales. In *Hymenasplenium*, on the other hand, rhizomes are creeping and dorsiventral (Hayata, 1927; Murakami, 1995). Stipes are non-articulate, generally with two back-to-back C-shaped vascular bundles uniting into an X-shape in the rachis (Kramer and Viane, 1990). The fronds are simple to decompound and the veins are forking, free or anastomosing. Sori are elongated and positioned along the veins and in many cases they are protected by indusia. Some species, such as *A. theciferum* have submarginal sori with an indusium fused with the lamina laterally and forming pouch-like structures (Holttum, 1966; Bellefroid *et al.*, 2010). Most *Asplenium* species have a chromosome base number of $x = 36$ (Manton, 1950) but other chromosome numbers have been found for tropical *Hymenasplenium* species ($x = 39$, $x = 38$) and for 'loxoscapoid' *Asplenium* species ($x = 35$) (Bellefroid *et al.*, 2010).

Many aspects of the anatomy of Aspleniaceae are discussed in some general works on plant anatomy (von Sachs, 1868; van Tieghem, 1886-1888; Strasburger, 1888; Strasburger, 1891), fern anatomy (Hofmeister, 1851; de Bary, 1877; Campbell, 1895; Pelourde, 1906; von Guttenberg, 1965; Ogura, 1972); fern leaf anatomy (Thomae, 1886; Hayata, 1927, 1930) and fern root anatomy (Lachmann, 1889; Rumpf, 1904; Pelourde, 1906; Schneider, 1996). Many authors also performed detailed anatomical studies of different organs or structures of Aspleniaceae such as leaves (Mitsuta *et al.*, 1980; Chaerle and Viane, 2004), rhizomes (Iwatsaku and Kato, 1975; Mitsuta, 1980), roots (Gifford, 1991; Schneider, 1997), reproductive structures (Oteo *et al.*, 1990), stomata (Van Cotthem, 1970a,b; Apostolakos and Galatis, 1999; Apostolakos *et al.*, 2009a,b), spores (Viane, 1992; Morbelli and Giudice, 2005), tracheids (Luna *et al.*, 2010; Schneider and Carlquist, 1999); others presented a comparative overview of the anatomy of different structures (Tardieu-Blot, 1932; Umikalsom, 1992; Van den heede, 2004).

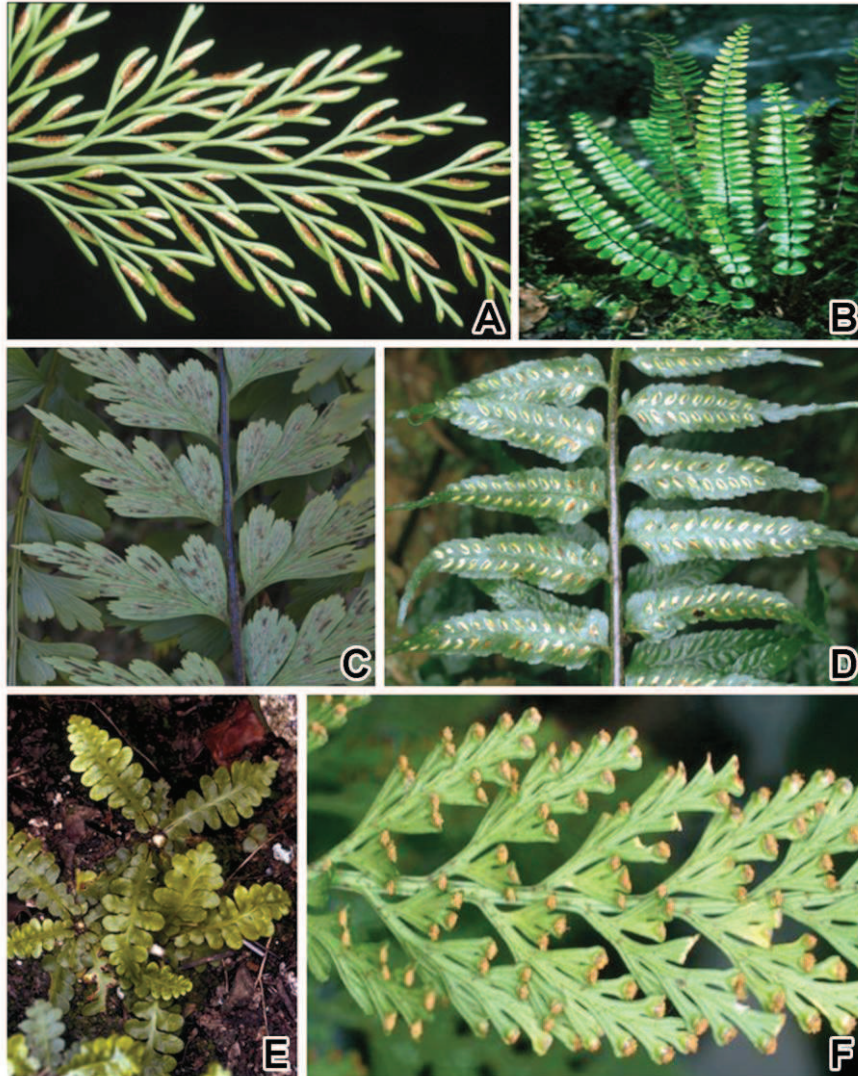


Figure 1.2: Aspleniaceae. A. *A. daucifolium*. B. *A. anceps*. C. *A. aethiopicum*. D. *A. elliotii*. E. *A. dalhousiae*. F. *A. theciferum*. (photos R. Viane)

Several systematic studies within the Aspleniaceae have focused on single morphological or anatomical aspects such as cytology (Cheng and Murakami, 1998), rhizome anatomy (Mitsuta *et al.*, 1980), roots (Schneider, 1997), spore ornamentation (Viane and Van Cotthem, 1977; Puttock and Quinn, 1980; Tryon and Lugardon, 1991; Viane, 1992), or vascular tissue (Khare and Shankar, 1989; Saiki *et al.*, 1989; Umikalsom, 1992). Recent phylogenetic studies based on *rbcL* and/or *trnL-F* nucleotide sequence data

usually confirmed the taxonomical significance of some of these anatomical characters. For example, the recognition of *Hymenasplenium* was based on its dorsiventral creeping rhizomes, unlike most other species within the Aspleniaceae family with radial, erect or short-ascending rhizomes (Hayata, 1927). Murakami (1995), Murakami *et al.* (1999), and Schneider (2004) showed that *Hymenasplenium* is the most basally diverged monophyletic group distantly related to any of the remaining species of Aspleniaceae. Furthermore, Rumpf (1904), Pelourde (1906), Schneider (1997) and Chaerle (2006) showed that the innermost layer of the root sclerenchyma sheath in some species only consist of six sclereids, and Schneider (2004) showed that these species formed a monophyletic group. Moreover, *Hymenasplenium* species are characterized by uniformly thickened root sclereids, whereas *Asplenium*-species have typical U-shaped thickened sclereids (Schneider, 1997).

1.4 PLANT CELL WALLS

Plant cell walls are crucial cell components and determine modes of development, specifically cell division, cell enlargement, cell adhesion, and cell differentiation processes (Keegstra, 2010). They are dynamic and hence often remodelled and reconstructed during growth and development, and in response to environmental stress or pathogen attacks. Materials obtained or isolated from cell walls are often commercially important, and are a source of functional food ingredients, industrial fibres, nutraceuticals and feed stocks for second-generation bio-fuels (Himmel *et al.*, 2007). They must be strong enough to maintain turgor pressure, but, on the other hand they must enable cell expansion. Therefore cells have the ability to alter their structure and composition, and hence their properties, during growth, development and maturation. Traditionally, they are classified as primary and secondary cell walls. Primary cell walls are deposited while the cell is still enlarging and any additional microfibrils laid down after cell expansion has ceased, form part of the secondary cell wall (Bailey and Kerr, 1935; Frey-Wyssling, 1978; Carpita and Gibeaut, 1993). Secondary cell walls can be further divided into those with lignified walls, which are the most common, including most sclerenchyma fibers and tracheary elements, and those with non-lignified cell walls. As most researchers only focus on either non-lignified primary cell walls or lignified secondary cell walls, little is known about non-lignified secondary cell walls. The latter include the thick walls of parenchyma cells in the cotyledons, and endosperms of many angiosperm seeds and cotton seed hairs (Harris, 2006).

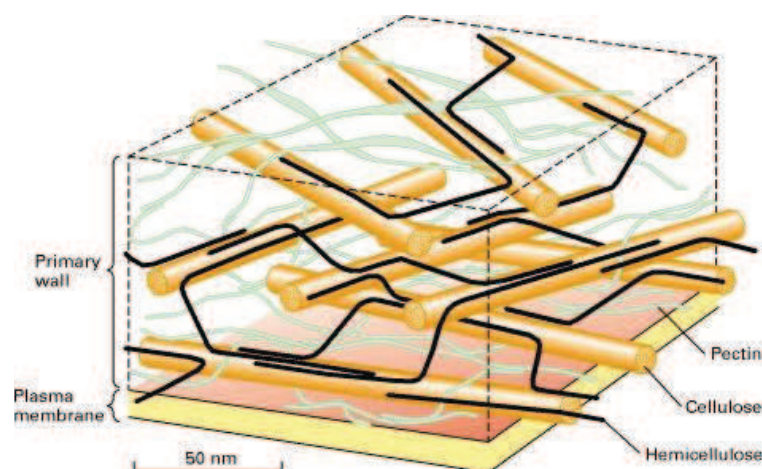


Figure 1.3: Schematic representation of a primary cell wall (Lodish *et al.*, 2000). Proteins not included.

Generally, the cell wall consists of two phases: a fibrillar phase of crystalline cellulose and a matrix phase of complex non-cellulosic polysaccharides (hemicelluloses and pectic polysaccharides) (Carpita and Gibeaut, 1993, Cosgrove, 2005). Structural proteins and glycoproteins, as well as phenolic compounds may also be present. Cellulose is the most stable of all of the cell wall polysaccharides and is made of parallel unbranched (1,4)-linked- β -D-glucans assembled into crystalline microfibrils (Cosgrove, 2005). Hemicelluloses are cellulose binding polysaccharides, which form a strong network together with cellulose (Cosgrove, 2005; Scheller and Ulkov, 2010) (Fig. 1.3). Their backbone resembles that of cellulose, but their structural modifications prevent most of them to form microfibrils by themselves. Most polysaccharides of the hemicellulose and pectic groups display extensive variants in terms of both glycosyl structure or polysaccharide modifications, such as methyl esterification or acetylation. Xyloglucans, xylans and mannans are three of the most abundant hemicelluloses. Xyloglucans are major components in most primary cell walls and are proposed to have a functional role in tethering the cellulose microfibrils together. They are highly diverse and this variation has been shown to be of taxonomical significance (O'Neill *et al.*, 2003). Xylans are the most abundant polysaccharides in nature after cellulose (Carpita and Gibeaut, 1993). In most plants they are abundant components of secondary cell walls associated with vascular tissues (Brett and Waldron, 1996), but they have also been detected in primary cell walls (Hervé *et al.*, 2009). Mannans are a complex set of hemicellulosic heteroglycans that can contain backbone glucosyl and/or side branch

galactosyl residues and are proposed to have storage and structural functions in cell walls (Scheller and Ulaskov, 2010). Callose is a general term for β -1,3-glucans which are found in a number of specific cell walls such as perforation plates of phloem sieve cells. However, some of this callose may be artefactual, since callose is formed by a variety of cells in response to wounding, and may be deposited on the sieve plates as plant material is harvested for study (Brett and Waldron, 1996). (1,3)(1-4)- β -glucans are known as mixed-linkage glucans which are important constituents of the cell walls of cereals and grasses of the Poaceae (Carpita and Gibeaut, 1993). Pectic polysaccharides are complex polymers forming hydrated gels. They are important factors of cell wall porosity and they are also the main component of the middle lamellae (Cosgrove, 2005). They are especially abundant in extendable primary cell walls and can be classified in three main groups of polysaccharides: homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (Willats *et al.*, 2001). Homogalacturonans are the main components of pectin and consist of 1,4-linked galacturonosyl residues. RG-I is a structurally distinct and heterogeneous set of polymers based on rhamnogalacturonan backbones with diverse arabinan, galactan and arabinogalactan side chains (Willats *et al.*, 2006; Albersheim, 2010), and the complex RG-II domain can be considered as a specific, elaborated form of homogalacturonan allowing boron-mediated cross-linking of homogalacturonan chains (Mohnen, 2008). Cell walls also contain a variety of proteins. Extensins are water soluble hydroxyproline-rich glycoproteins which may be able to form cross-links in the cell wall (Brett and Waldron, 1996). Another important class of cell wall proteins are arabinogalactan-proteins (AGPs), which have a high carbohydrate content. Many AGPs are soluble proteins, while others possess lipid tails anchoring them to the outer surface of the plasma membrane. Their occurrence is developmentally regulated, but their specific functions remain unclear (Albersheim *et al.*, 2010). Many secondary cell walls contain lignins which are defined as complex polymers resulting from the oxidative polymerization of three types of hydroxyl-cinnamyl alcohol precursors resulting in *p*-hydroxyphenyl, guaiacyl and syringyl lignin units (Boerjan *et al.*, 2003).

Two types of cell walls have been distinguished in higher plants. Type I cell walls are found in the majority of plants, including gymnosperms, dicots, and non-commelinid monocots, whereas type II walls are found in commelinid monocots (Carpita and Gibeaut, 1993; Carpita, 1996). Cellulose is the main polysaccharide in both types. In type I cell walls xyloglucan is the main hemicellulosic polysaccharide. This cellulose-xyloglucan framework is embedded in a matrix of pectic polysaccharides, homogalacturonan and rhamnogalacturonans I and II. Type II cell walls, on the other hand, are characterized by low levels of xyloglucan, pectins and structural proteins, and glucuronoarabinoxylans are

the main cross-linking polysaccharides. One group within the commelinid monocots, the Poales, have walls that contain mixed-linkage glucan ((1→3)(1→4)-β-D-glucan) (Carpita, 1996). For detailed reviews on general cell wall architecture and polymer structure, I refer to Bacic *et al.* (1988); Carpita and Gibeaut (1993), Brett and Waldron (1996), O'Neill and York (2003), Cosgrove (2005), Harris (2005), Mohnen (2008), Scheller and Uliskov (2010), and Albersheim *et al.* (2010).

1.5 CELL WALLS OF FERNS AND LYCOPHYTES

The structure of cellulose is conserved across the plant kingdom, but the structures and relative amounts of other cell-wall components are highly variable, not just between plant species but also between organs, within tissues and even within an individual cell wall (Willats *et al.* 2001; Popper and Fry, 2004a; Harris, 2005; Popper, 2008; Popper and Suohy, 2010; Sørensen *et al.*, 2010; Lee *et al.*, 2011). Compared with the walls of other vascular plants, little is known about the composition of fern and lycopphyte cell walls (Popper, 2006).

Biochemical studies of primary cell walls (Popper and Fry, 2003, 2004a) have shown that xyloglucan, a major cross-linking polysaccharide in seed plants (Carpita and Gibeaut, 1993; O'Neill and York, 2003), also occurs in seedless tracheophytes (pteridophytes) as well as in bryophytes. An unusual sugar, 3-O-methylrhamnose, was extracted from cell walls of charophycean algae, liverworts, mosses, an anthocerot and homosporous lycopod, but was not detected in the cell walls of a heterosporous lycopod, nor in ferns, gymnosperms or spermatophytes (Popper *et al.*, 2004b). Furthermore, the monosaccharide residue 3-O-methylgalactose is a major component of cell walls of homosporous and heterosporous lycopphytes (Popper and Fry, 2001) and the presence of this component is a synapomorphy for the monophyletic lycopphyte clade because this sugar is almost absent in primary cell walls of other land plants. Primary cell walls of pteridophytes and spermatophytes also present quantitative differences in sugar composition (Popper and Fry, 2004a). In contrast to the primary cell walls of seed plants, fern, lycopphyte and bryophyte cell walls contain high amounts of mannose (Bailey and Pain, 1971; Popper and Fry, 2004a; Popper, 2006). Eusporangiate ferns and bryophyte primary cell walls typically contain more mannose than primary cell walls of leptosporangiate ferns (Popper and Fry, 2003, 2004a). Popper and Fry (2003) presented evidence that pectic homogalacturonan is present in fern cell walls. Pectic rhamnogalacturonan-I, on the other hand, has not been reported to occur in fern nor lycopphyte cell walls. Rhamnogalacturonan II (RG-II), a structurally complex borate-

linked pectic polysaccharide present in the primary walls of gymnosperms and angiosperms (O'Neill *et al.*, 2004), has been found to occur in pteridophytes (Matsunaga *et al.*, 2004). Recently, mixed-linkage (1→3)(1→4)-β-D-glucan (MLG) molecules were discovered in cell walls of *Equisetum* (Sørensen *et al.*, 2008; Fry *et al.*, 2008). Until recently, MLGs had only been reported for commelinoid monocots (Carpita and Gibeaut, 1993). This unexpected presence of MLG in *E. arvense* was discovered only because the cell walls of a large number of phylogenetically diverse plants were sampled. Analysis of isolated secondary cell walls have not been undertaken, but galactoglucomannans and 4-O-methylglucuronarabinoxylans have been isolated from *Osmunda* and *Pteridium* stipes (Timell, 1962a,b; Bremner and Wilkie, 1966, 1971).

In parallel with biochemical investigations, recent immunocytochemical studies have revealed that polysaccharide and glycoprotein epitopes present in angiosperm cell walls (Knox, 1997; Willats and Knox, 2003) also occur in pteridophytes and lycophytes (Ligrone *et al.*, 2002; Kremer *et al.*, 2004; Carafa *et al.*, 2005). Immunolabelling experiments using the monoclonal antibodies LM10 and LM11, both recognising xylan-epitopes, showed that xylan-polymers are present in the secondary cell walls of vascular tissues in all extant tracheophytes (Carafa *et al.*, 2005). However, only few *in situ* immunolabelling experiments have been undertaken to assess the distribution of specific polymers in fern or lycophyte cell walls.

In conclusion, the present literature shows that changes in cell wall molecules are likely to have been fundamental to the emergence, divergence and survival of extant land plants. Unfortunately, our understanding of the origins of cell walls and the changes in wall structure that occurred during the evolution of land plants is limited by the lack of information on wall composition and architecture in non-flowering plants.

1.6 GENERAL AIMS AND OUTLINE

During a preliminary anatomical study of roots, rhizomes, and leaves of *Asplenium anisophyllum* and related species (Leroux *et al.*, 2005), we found different cell wall-related root features of which little or no histological details were known. The initial aims of this PhD study were (1) to subject these anatomical features to detailed histological analyses, (2) to study their potential taxonomical significance within Aspleniaceae, and (3) to evaluate the hypotheses of Schneider (1997). During these studies considerable attention has been given to cell wall composition and structure. Fern cell walls have not been studied extensively, and hence there is no clear understanding of how they conform to models of cell wall structure developed for angiosperms. Moreover, the recent discovery of mixed-linkage glucan in *Equisetum* shows that large-scale surveying of cell walls across the plant kingdom may yield valuable new data contributing to our knowledge of cell wall evolution and diversity. Therefore, we broadened our research interests towards (fern) cell wall biology.

In order to allow clear presentation of the findings of a variety of studies, this thesis is divided into four parts:

PART ONE (Introduction & technical papers) comprises the current introductory chapter (CHAPTER 1) and two technical papers. In CHAPTER 2, we describe an easy, quick and low cost technique for oriented embedding of thin structures using glycol methacrylate resin and self-constructed re-usable embedding tools [A sandwich-embedding method for oriented sectioning]. A preparative method for three-dimensional visualisation of plant structures, organs and tissues using X-ray CT is presented in CHAPTER 3 [A new method for studying plant structures using X-ray computed tomography].

PART TWO (Aspleniaceae: root anatomical features) is dedicated to the histological study of cell wall-related root anatomical characters in Aspleniaceae and addresses the following research questions:

- Are the yellow-brown coloured cell walls of the sclerenchyma sheath in roots (Fig. 1.4A) lignified, and how does their structural variation fit with the current knowledge on phylogenetic relationships within the family? [CHAPTER 4 - Histogeny, structure, composition and taxonomical significance of the sclerenchyma sheath in Aspleniaceae roots (Polypodiales)]

- Outer root cortical cells of some *Asplenium* species develop helical cell wall thickenings (Fig. 1.4B): what is their structure, cell wall composition and taxonomical significance? [**CHAPTER 5 - Non-lignified helical cell wall thickenings in root cortical cells of Aspleniaceae (Polypodiales): histology and taxonomical significance**]
- The inner cell wall surface of the outer cortex cells of some *Asplenium* roots is often studded with intracellular cell wall projections (Fig. 1.4C): what is the nature and origin of these structures? [**CHAPTER 6 - Ultrastructure and composition of cell wall appositions in the roots of *Asplenium* (Polypodiales)**]

PART THREE (A focus on fern cell walls) assembles our studies focussing on fern cell wall composition. [**CHAPTER 7 - An antibody-based analysis of fern cell walls confirms cell wall diversity and taxonomic patterns in the plant kingdom**]. The aim of this study was to investigate the potential tissue-specific and taxonomically significant distribution of cell wall polymers and their structural variants. Some of the interesting findings of this study were subjected to detailed histological studies:

- A specialised cell wall lines carinal canals in *Equisetum* and a detailed histological study was carried out to investigate the (ultra)structure and development of carinal canals, as well as to the molecular composition of their cell wall linings [**CHAPTER 8 - A specialised cell wall lines the carinal canals in *Equisetum ramosissimum* Desf. (Equisetaceae, Pteridophyta)**].
- The intercellular spaces of the cortical parenchyma in many *Asplenium* petioles are packed with wart-like and filamentous projections (Fig. 1.4E). What is their structure and composition, and how do they develop? [**CHAPTER 9 - Intercellular Pectic Protuberances in *Asplenium aethiopicum*: new data on their composition and origin**]
- Even though collenchyma has not been reported in leptosporangiate ferns, we found comparable tissues in some *Asplenium* petioles (Fig. 1.4D). What is their structure and composition and can we refer to them as collenchyma tissues? [**CHAPTER 10 - Collenchyma in ferns? Perspectives from a histological study of collenchymatous cell walls in *Asplenium rutifolium* (Polypodiales)**]

PART FOUR (General discussion and future studies) puts forward the general discussion and future studies along with some preliminary results.

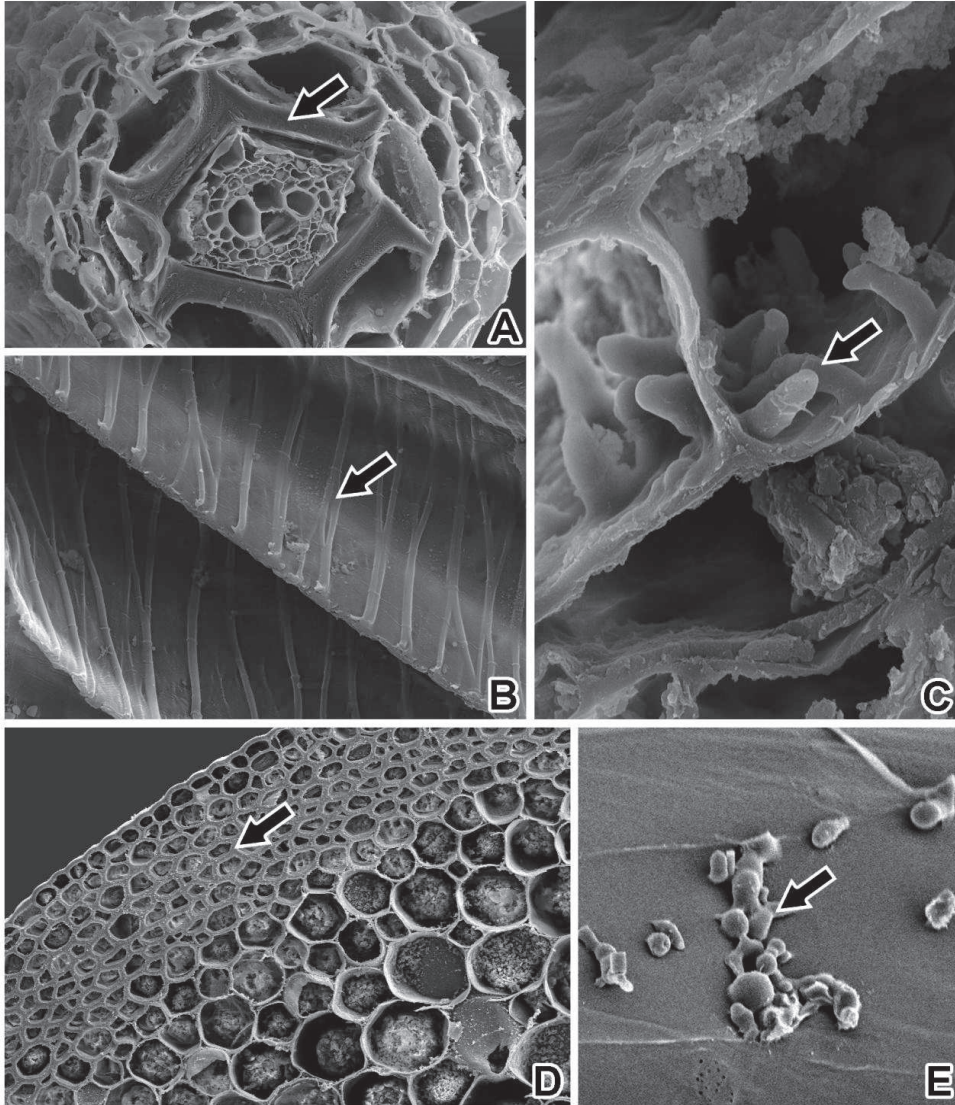


Figure 1.4: Cell-wall related features in Aspleniaceae. **A.** Scanning electron micrograph showing sclereids (arrow) surrounding the stele of an *A. aethiopicum* root. **B.** Helical cell wall thickenings in the outer root cortex of *A. nidus*. **C.** Cell wall appositions in the outer root cortex of *A. elliotii*. **D.** Collenchymatous tissue in the petiole of *A. rutifolium*. **E.** Intercellular pectic protuberances in *A. aethiopicum* petioles. (photos: [A-D] O. Leroux and A. Bagniewska-Zadworna, [E] A. Vrijdaghs)

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2

A sandwich-embedding method for oriented sectioning

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Summary

High-quality sections are indispensable for many scientific studies. Most published methods are often time-consuming or require special devices. We present an easy, quick and low-cost method for oriented embedding of thin structures using glycol methacrylate resin and self-constructed, reusable embedding tools made of overhead transparencies. This technique allows for more flexibility in orientation than other methods, enabling precise transverse, longitudinal and even oblique sectioning.

2.1 INTRODUCTION

Many biological studies rely on good-quality sections. The orientation of the object is of major importance to obtain highly informative images when, for example, examining the ontogeny of plant structures, producing three dimensional reconstructions, analyzing gene expression patterns, or performing immunolocalization of proteins. When perfectly transverse sections are required, the skewed orientation of the embedded specimen regularly causes artefacts complicating the analysis or leading to sections unsuitable for publication. The key to perfect sections often lies in finding an appropriate embedding method. Several publications propose methods for oriented embedding for light and electron microscopical purposes (Feder & O'Brien 1968, Nanchahal & Watts 1983, Nelson & Woods 1996, Beeckman & Viane 2000, Trancik *et al.* 2001, Hill *et al.* 2002). Unfortunately, some of these methods are either time consuming, complicated, or require expensive tools. However, the method described by De Smet *et al.* (2004) using safe-lock tubes is an easy technique for transverse sectioning. For this method, pipette tips and safe-lock tubes are used to create moulds with a conical cavity to embed cylindrical samples with a diameter above 500 μm . Narrower samples ($\varnothing < 500 \mu\text{m}$) should then be embedded in self constructed flat moulds made of overhead transparencies and double-sided tape (Beeckman & Viane 2000). Although the construction of such flat moulds is time-consuming, the efficiency of this technique has been demonstrated using *Arabidopsis thaliana* (L.) Heynh. roots (Beeckman & Viane 2000).

Besides transverse and longitudinal sections, oblique sections may also provide useful information. According to Gifford (1991), they are indispensable for studying the merophyte development in root apical meristems of ferns and fern allies. To our knowledge, no modern method for embedding material at well-defined angles has been published to date.

Consequently we present a new, easy, quick and low cost technique for the oriented embedding of thin structures using glycol methacrylate resin and self-constructed re-usable embedding tools. This method allows for more flexibility in orientation, because it enables accurate transverse, longitudinal or oblique sectioning of thin randomly shaped structures with a thickness or diameter less than 2 mm.

2.2 MATERIALS AND METHODS

2.2.1 Materials

To demonstrate the flexibility of our technique, we processed rhizome scales of , adventitious roots of *A. anisophyllum* Kunze, root tips of *A. elliottii* C.H. Wright, and protocorms of *Epipactis helleborine* (L.) Crantz for embedding in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany).

2.2.2 Methods

Preparing samples for embedding

Protocorms originated from soil incubated seeds according to Van der Kinderen (1995). Root and scale samples were fixed overnight in FAA (50% v/v ethanol, 5% v/v acetic acid and 5% v/v commercial formalin in distilled water). Dehydration was performed using 30%, 50%, 70%, 85% and 94% ethanol. After the last alcohol step, the tissue was infiltrated using a mixture of Technovit 7100 liquid (2-hydroxyethylmethacrylate) and Hardner I (dibenzoylperoxide) which was diluted in advance to 30%, 50%, and 70% with ethanol 94%. Once the tissues were infiltrated with each of these solutions (1 day each), they were incubated in 100% infiltration liquid at 4 °C for 2 days. Specimens are then ready for embedding.

Embedding for transverse or oblique sections

For transverse or oblique sections of thin randomly shaped structures with a thickness or diameter less than 2 mm we use the following sandwich-embedding method. Prior to embedding, a template in the form of the median longitudinal section of a safe-lock tube is made from a commercially available overhead transparency (Fig 2.1a). Such a template can easily be cut using a median longitudinally sectioned safe-lock tube as reference. Next to a template, a support transparency is needed for the embedding procedure. A small drop of fresh embedding solution, made by mixing infiltration solution with Hardner II (dimethylsulfoxide), is put on the support transparency. After placing the sample in the embedding solution (Fig. 2.1a), the template is put on top of the material (Fig. 2.1b), and the space between both transparencies is completely filled with embedding solution (Fig. 2.1c). In this stage, the final orientation of the material can be changed using preparation needles or by moving the template. For oblique sectioning, the desired angle can be drawn on the template prior to embedding. When the template

is put onto the material, small needles can be used to orientate the sample according to the drawn lines on the template.

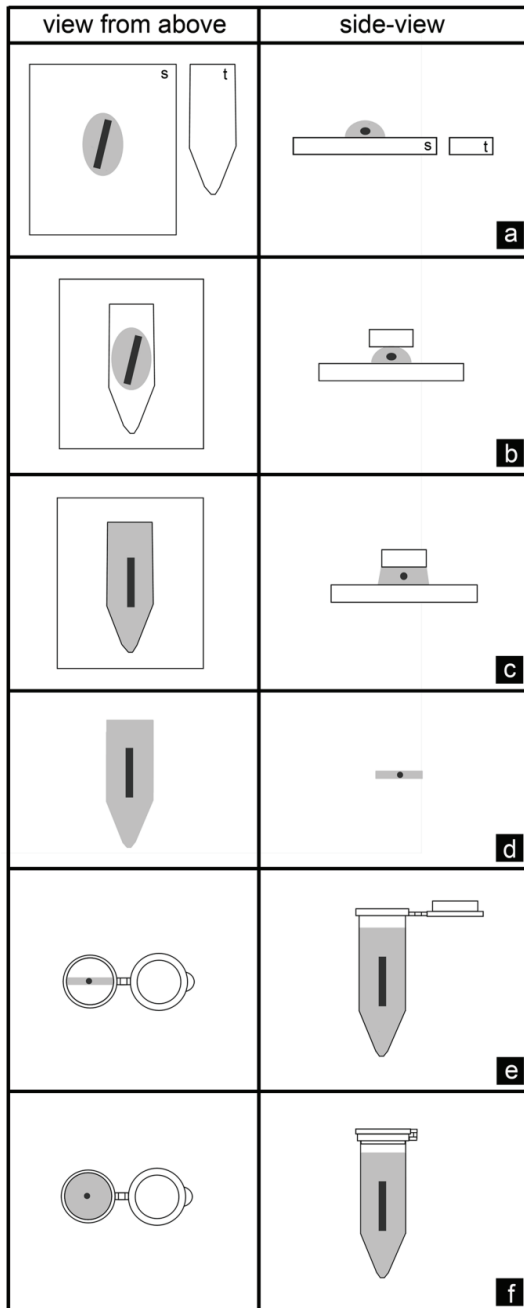


Figure 2.1: Schematic illustration of the sandwich-embedding method: example for transverse sections. (a) The sample is put on a drop of embedding solution on the supporting transparency; (b) A template is placed on the supporting transparency; (c) Embedding solution is added until space between template and supporting transparency is completely filled. Using preparation needles, the sample can be orientated prior to polymerization; (d) Removal of the supporting transparency and template after polymerization; (e) The plastic plate is fitted tightly in a safe-lock tube; (f) The safe-lock tube with plastic plate is filled with freshly made embedding solution, closed and left aside for overnight polymerization. Grey: embedding solution, s: supporting transparency, t: template.

After polymerization at room temperature or in a laboratory oven at 40 °C, both the template and the supporting transparency are removed leaving a plastic plate including the sample (Fig. 2.1d). This plate is tightly fitted into a safe-lock tube. Because the plastic plate has the same shape as well as the maximally possible inside diameter of the safe-lock tube, a perfectly vertical orientation is guaranteed (Fig. 2.1e). Next, the safe-lock tube containing the plastic plate is filled with freshly made embedding solution (Fig. 2.1f). After closure of the tube, the resin is left to cure for 24 h at room temperature or in a laboratory oven at 40 °C. Because the plastic plate is extremely moisture-sensitive, immediately proceed to the second embedding or keep the plates moisture-free. Cylindrical samples with a diameter larger than 2 mm can be embedded using the method previously described by De Smet *et al.* (2004).

Embedding for longitudinal sections

For longitudinal sectioning of very thin structures we use the sandwich technique described above using templates fitting in the mould cavities of a Teflon embedding mould (Histoform S, Heraeus Kulzer, Wehrheim, Germany). After pre-embedding, polymerization and removal of the transparencies, the obtained plastic plates are fixed horizontally on the bottom of the mould cavities using double-sided tape as in Beeckman & Viane (2000). However, when samples have a diameter or thickness of more than 2 mm, they can be immediately fixed on the bottoms of the mould cavities with double-sided tape. Subsequently, embedding solution is poured into the cavities, followed by polymerization for 24 h at room temperature or in a laboratory oven at 40 °C.

Post embedding

After polymerization, the safe-lock tubes were removed using a Top Craft TMW-40F rotary tool equipped with a DREMEL diamond grinding disc. When the Histoform mould was used, the polymerized blocks were extracted from the mould and mounted on Histoblocks (Heraeus Kulzer, Wehrheim, Germany) with a fast-curing resin (Technovit 3040 kit, Heraeus Kulzer, Wehrheim, Germany). Sections of 5 µm were made with a microtome (Minot 1212, Leitz Wetzlar, Germany) equipped with a holder for disposable Superlap Knives (Adamas Instrumenten, The Netherlands). They were collected separately on water drops placed on Vectabond-coated slides (Vector Laboratories, Burlingame, CA, USA). Subsequently, slides were dried on a hot plate at 40°C, stained with a 0.05% [w/v] aqueous solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) and 0.1% [w/v] Na₂B₄O₇ and mounted in DePeX (Gurr, BDH

Laboratory, UK). Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

2.3 RESULTS AND DISCUSSION

Controlling sample orientation while embedding is often needed when perfect transverse, oblique or longitudinal sections are required. Frequently, resin blocks or knife holders are adjusted to obtain certain orientations of embedded material. However, some samples are so small that orientation using microtome accessories is not possible, explaining the need for appropriate embedding methods. Using this sandwich-embedding method, material can be orientated with precision prior to final embedding, using a stereomicroscope if necessary. As only a few protocorms were available, this method enabled high quality transverse sections after embedding only one sample (Fig. 2.2a). We also obtained transverse sections of fern scales (Fig. 2.2b) which tend to curl in the embedding solution. In this case, the sandwich-embedding method presses the scale between the transparencies, ensuring a completely stretched sample for oriented embedding. We obtained good transverse (Fig. 2.3), longitudinal (data not shown) and oblique (data not shown) sections of the root apical meristem of *A. elliotii*.

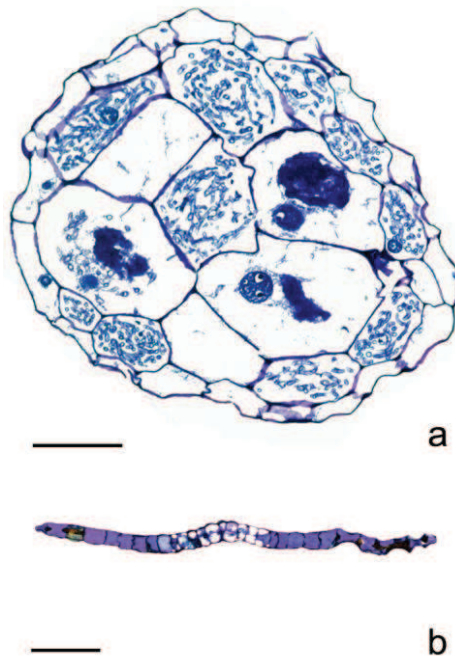


Figure 2.2: Light microscopic images of transverse sections through a 29-month soil incubated protocorm of *Epipactis helleborine* (L.) Crantz (a) and through a rhizome scale of *A. aethiopicum* (b). Scale bars: 100 μm .

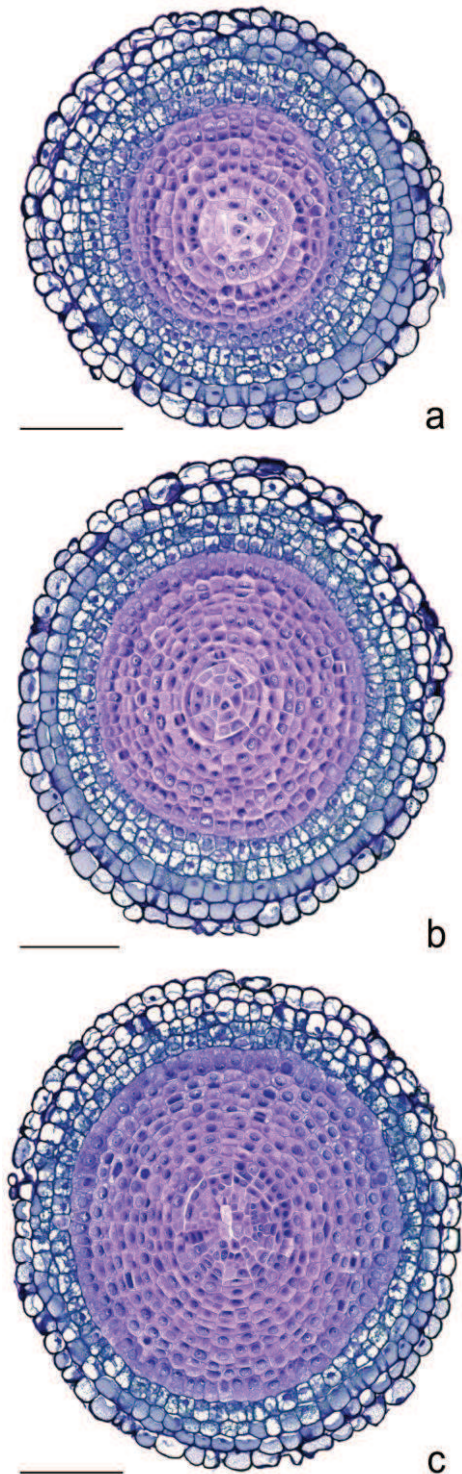


Figure 2.3: Light microscopic images of transverse sections through a root tip of *A. Elliottii*. (a) Section showing the root apical cell (centre). (b, c) Sections made just above the root apical cell showing early stages of vascular development (centre). Scale bars: 100 μm.

When this method is used, badly orientated specimens seldom occur, which is important when only a few samples are available. Furthermore, embedding of more than one sample is possible. The self constructed templates, as well as the supporting transparencies are re-usable and different sizes of safe-lock tubes can be used for this method. Although pre-embedding could result in uneven hardness of the resin or fracture-areas due to the two-step embedding, we did not experience such problems.

2.4 CONCLUSIONS

In summary, this method minimizes some of the disadvantages related to the use of traditional procedures as (1) it allows precise orientation, (2) it is low cost and quick, (3) for transverse and oblique sectioning, no expensive moulds or tools are needed, (4) the templates are re-usable and (5) a wide variety of samples with a thickness or diameter less than 2 mm can be embedded.

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3

A new preparation method for studying fresh plant structures using X-ray computed tomography

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Summary

Since the development of X-ray computed tomography as a medical diagnostic tool, it was subsequently adapted and extended for many scientific applications, including plant structure research. As for many biological studies, sample preparation is of major importance to obtain good-quality images. Therefore we present a new sample preparation method which includes heavy metal *en bloc* staining and critical point drying. This technique enhances the contrast of all tissues, prevents artefacts such as tissue compression, and requires no embedding.

3.1 INTRODUCTION

As in many biological disciplines, advances in microscopy have created new opportunities to study plant structures. Since the development of X-ray computed tomography (CT) for medical purposes, it was extended and adapted for a wide range of industrial and scientific applications (e.g. Hounsfield, 1980; Takeda, 2005; Tafforeau *et al.*, 2006; Mendoza *et al.*, 2007; Westneat *et al.*, 2008).

Due to its non-destructive nature and ability to overcome the imaging constraints imposed by the soil medium, X-ray CT has been successfully used to examine plant root architecture in situ (Pierret *et al.*, 1999; Hamza *et al.*, 2001; Gregory *et al.*, 2003; Kaestner *et al.*, 2006; Perret *et al.*, 2007), but with spatial resolutions incapable of visualizing the internal structure of the root. In botany X-rayCT has not only been used to study fossilized material, such as the trunks of Cycadeoidea (Pika-Biolzi *et al.*, 2000), the fossil fruits of *Palaeorhodomirtus subangulata* (DeVore *et al.*, 2006), the Cretaceous seeds (Friis *et al.*, 2007) and flowers of *Potomacanthus* (von Balthazar *et al.*, 2007), but also to investigate the density of extant woods (Taylor *et al.*, 1984; Funt & Bryant, 1987; Lindgren, 1991; Fromm *et al.*, 2001), the inflorescence of *Leucospermum tottum*, the fruit of *Syagrus flexuosa* and the wood of *Quercus robur* (Stuppy *et al.*, 2003). In the majority of the fresh samples studied (e.g. Stuppy *et al.*, 2003; Mendoza *et al.*, 2007), a good spatial resolution of organ systems was achieved, even though tissue or cellular level detail was not fully resolved. As mentioned by Stuppy *et al.* (2003), differences in X-ray attenuation in plant tissues and organs depend primarily upon the thickness and consistency of cell walls and cell contents. Therefore, the best results are obtained from samples that contain different types of tissues with dissimilar densities. Previous studies using X-ray CT on living plants obtained good results for wood, but were less successful with samples or tissues with higher water content (Stuppy *et al.*, 2003). Woody tissue consists mainly of dead, empty cells with lignified secondary walls, whereas fresh parenchymatous tissues have thin primary walls and high water content. The properties of cell walls are greatly affected by the presence of phenolic compounds such as lignins. In secondary cell walls lignins replace water, transforming the wall into a hydrophobic and denser environment (Brett & Waldron, 1996). Due to their higher density, secondary cell walls are more easily detected with X-ray CT in comparison to soft parenchymatous tissues.

The alternative method to obtain three-dimensional (3D) views of fresh plant structures by combining serial sections has problems of misalignment and/or missing sections. Moreover, when sectioning strongly sclerified plant materials, the tissues frequently tear apart due to tissue heterogeneity. Next to these methodological constraints, producing 3D views by processing serial sections is labour intensive. Nuclear

magnetic resonance proved to produce excellent results in several studies (Möller *et al.*, 2000; Mill *et al.*, 2001; Glidewell *et al.*, 2002), but has a limited spatial resolution of 10–100 μm (Lee & Kim, 2008). Other methods with submicrometre resolution, such as confocal laser scanning microscopy and electron tomography, are restricted in use because they require thin, small and at least semi-transparent objects (Lee & Kim, 2008). Complementary tomographic techniques, such as X-ray fluorescence, have been developed in order to study the chemical properties of samples (e.g. Pereira *et al.*, 2007; De Samber *et al.*, 2008). As biological materials are composed largely of water and therefore do not often produce usable imagery because of insufficient contrast, phase-contrast X-ray tomography has recently been used successfully to visualize the internal structure of biological samples (Takeda, 2005; Cloetens *et al.*, 2006; Friis *et al.*, 2007; Jakubek *et al.*, 2007; Westneat *et al.*, 2008). We strongly believe that the rapidly evolving technology of X-ray CT will allow many researchers to study biological material at higher resolutions, up to nanometre scale. However, as for all histological methods, sample preparation of fresh tissues is of major importance to obtain good results. Therefore, we present a new technique to process millimetre-sized freshly fixed plant samples for X-ray CT, which includes heavy-metal staining and critical point drying.

3.2 MATERIAL AND METHODS

3.2.1 Material

To demonstrate our preparation method we processed fresh root samples of *Asplenium theciferum* (Kunth) Mett. (EB238 collected by E. Bellefroid in Zimbabwe, Bvumba Mts, Bunga Forest). Roots were cut into pieces of approximately 5-mm long and were fixed overnight, either in FAA (50% v/v ethanol, 5% v/v acetic acid and 5% v/v commercial formalin in distilled water), or in 2% paraformaldehyde [w/v] and 2.5% [v/v] glutaraldehyde in a cacodylate buffer 0.1M pH 7.4 for 24 h at 4°C. During the course of this PhD *Equisetum ramosissimum* stems have been studied using X-ray CT and these results are shown in Figures 3.2 and 3.3.

3.2.2 Light microscopy

After fixation samples were dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) following Leroux *et al.* (2007) [CHAPTER 2]. Transverse sections of 3 μm were cut with a Microm HM360 microtome (Microm International GmbH, Walldorf, Germany) equipped with a holder for glass knives, dried

on Vectabond-coated (Vector Laboratories, Burlingame, CA, USA) object glasses, stained with an aqueous 0.05% [w/v] solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% [w/v] $\text{Na}_2\text{B}_4\text{O}_7$ and mounted in DePeX (Gurr, BDH Laboratory, Poole, UK). Sections were observed with a Nikon Eclipse E600 microscope using a Plan-Apochromat 20 $\times$ /0.4 objective, providing a theoretical resolution of 0.69 μm (Murphy, 2001), and equipped with a Nikon digital camera DXM1200.

3.2.3 X-ray computed tomography

After fixation samples were post fixed in 2% osmium tetroxide, dehydrated to 50% ethanol and stained en bloc with 2% [w/v] uranyl acetate in 50% ethanol for 4 h at room temperature. After washing thoroughly, the samples were dehydrated in an ethanol series (70%, 85%, 94% and 100% twice). Subsequently, they were rinsed three times with liquid CO_2 and critical point dried using a CPD 020 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). They were stored in microporous specimen capsules kept in silica gel. Some samples were dehydrated to 70% ethanol without en bloc staining and critical point drying. We also processed roots for embedding in Paraplast (Sigma, St. Louis, MO, USA) according to Ruzin (1999).

Samples (either Paraplast embedded, air-dried or critical point dried) were glued in upright position on the surface of a metal stub using nail polish and scanned at the centre for CT (UGCT) of Ghent University (Masschaele *et al.*, 2007). The CT scanner is an in house developed very high-resolution CT (VHRCT) scanner, also called nanoCT scanner. The systems main components are a nanofocus X-ray tube (Feinfocus Inc., Garbsen, Germany), a high-resolution charge-coupled device (CCD) camera with fiber optic taper (Photonic Science, Robertsbridge, UK) and a sample manipulator which can move the samples with 6 degrees of freedom. The contrast of the CT data depends not only on the voltage setting of the tube but also on the spectral sensitivity of the X-ray imaging detector. Working at 50 kV turned out to be the best setting to reach a sufficient X-ray flux, the highest achievable spatial resolution and a good contrast in biological samples. The detector was used in binning mode, grouping 8×8 pixels into a super pixel of $76 \times 76 \mu\text{m}$. We scanned in nanoCT mode, with a focal spot size of 900 nm. We applied a magnification factor of 50 resulting in a voxel size of 1.5 μm . Because of the Nyquist theorem we can state that the spatial resolution of the images is two times the voxel size, thus 3 μm . Between 1000 and 2000 radiographs were taken per CT scan while rotating the samples from 0° to 360°. The exposure time per projection image was 500 ms. All data were processed from raw into CT slices with Octopus. Octopus is a user friendly, system independent, reconstruction package developed by XRayLAB (<http://www.xraylab.com>).

After the reconstruction we obtained over 300 cross-sections through the sample volume. VGStudio Max was used to create the 3D renderings (<http://www.volumegraphics.com>). All X-ray images were inverted using Adobe Photoshop CS2 software.

3.3 RESULTS AND DISCUSSION

Attempts to obtain good results using fixed roots dehydrated to 70% ethanol failed due to tissue compression and the detachment of the vascular cylinders after the rupture of the endodermal radial cell walls (data not shown). By saturating the scan chamber with 70% ethanol, we were able to reduce tissue compression to some extent after several trials, but vascular cylinders were always partly or completely detached and compressed (Fig. 3.1A). When scanning samples embedded in Paraplast, the contrast was inferior due to the decreased density heterogeneity (data not shown). Because the embedding medium surrounding the samples was scanned and imaged as well, the external surface of the samples cannot be studied together with the internal structure. By using our improved method we were able to study the anatomy of *Asplenium* roots without encountering the problems mentioned above. The *en bloc* staining was previously used by Ananda *et al.* (2006) to stain liver tissue. They tested several contrasting stains and have shown that an increase of immersion time in uranyl acetate improved contrast. They obtained a maximum contrast after 7 h of incubation in 2% uranyl acetate. We achieved good contrast after 3–5 h of incubation. However, the use of new contrast-enhancing techniques, such as phase contrast X-ray tomography (Takeda, 2005; Friis *et al.*, 2007; Jakubek *et al.*, 2007), will probably decrease the need for the expensive and toxic heavy metal staining. Our results were comparable notwithstanding the different fixation protocols we used.

The main improvement to the existing technique (Ananda *et al.*, 2006) is the extra application of critical point drying. After critical point drying the tissues are completely dehydrated and have a significantly increased density heterogeneity. We stress the importance of critical point drying as fresh tissues with high water content have often been difficult to visualize using X-ray CT (Stuppy *et al.*, 2003). Furthermore, critical point drying prevents shrinkage of soft parenchymatous tissues during the tomography procedure.

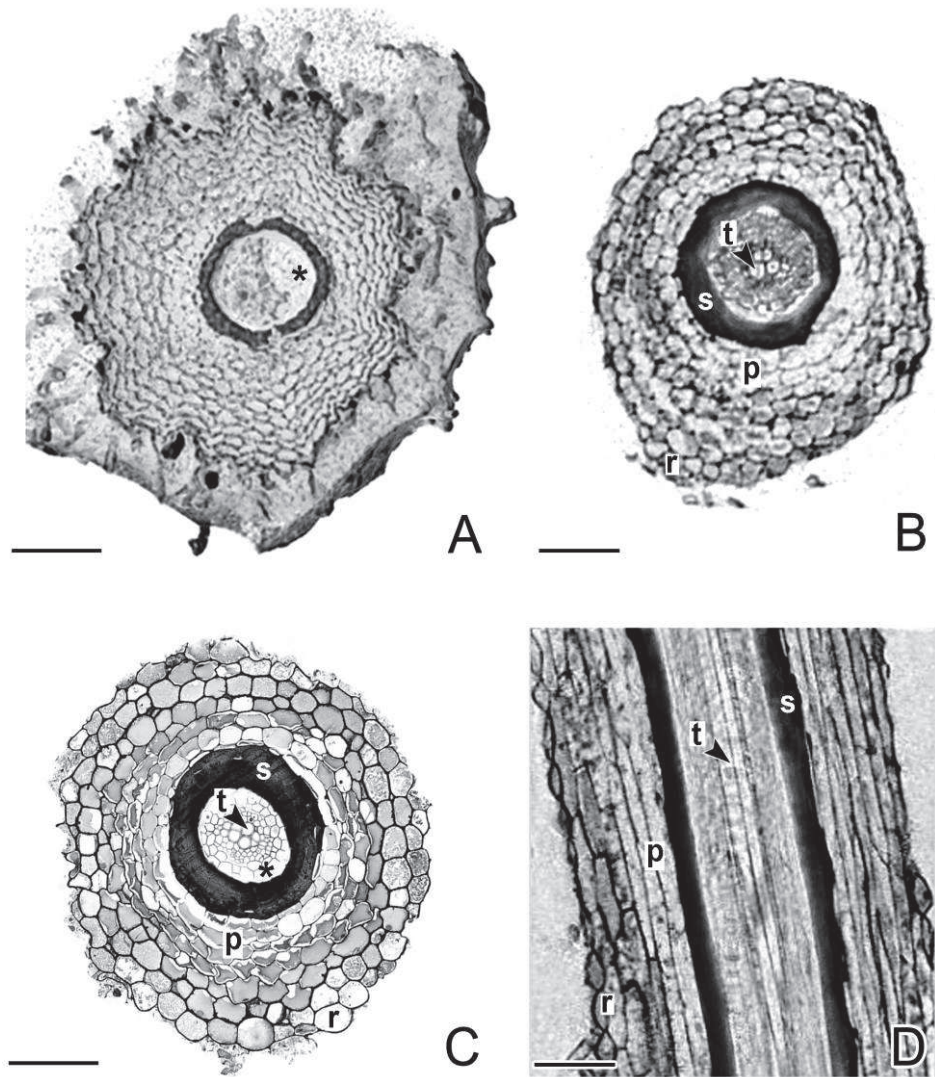


Figure 3.1: Images of *Asplenium theciferum* (EB238) roots. (a) X-ray CT image showing a cross section obtained after scanning a fixed sample in an ethanol saturated scan chamber. Tissue compression is obvious and the vascular cylinder is detached (*). (b) X-ray CT image showing a cross section obtained after critical point drying and *en bloc* staining of the sample. No tissue compression occurred and the vascular cylinder remained intact. (c) Transverse microtome section. As in figure 1B, no tissues are compressed or collapsed. (*) shows a detached endodermis, a frequently encountered artefact when sectioning fern roots. (d) X-ray CT image showing a longitudinal section. All tissues, including the vascular cylinder, remained intact. r: rhizodermis, p: parenchyma, s: sclerenchyma, t: tracheids. Scalebars: 150 µm.

Figure 3.1B shows an X-ray CT image of an *Asplenium theciferum* root after heavy metal staining and critical point drying. No tissue compression occurred and the vascular cylinder remained intact. A median longitudinal section of the same root is depicted on figure 3.1D. The rhizodermis, cortex parenchyma, cortex sclerenchyma and the vascular tissue can easily be distinguished. We scanned several samples, including younger roots, and never observed detached or compressed vascular cylinders. Figure 3.1B shows a transverse microtome section of an *Asplenium theciferum* root. Although the resolution of microtome sections (Fig. 3.1C) is currently higher than that obtained by X-ray CT, microtomy is labour-intensive and during sectioning tissues are often damaged. When making longitudinal sections of *Asplenium* roots tissues frequently tear apart due to large differences in cell wall resistance (hardness). In general, making 3D reconstructions using this traditional method can be problematic, whereas other methods, as the one presented here, can lead to satisfying results in a shorter period of time.

Figure 3.2 shows the comparison of SEM imaging versus 3D reconstruction of X-ray CT data of the surface of an *Equisetum ramosissimum* stem and virtual slices through the same stem are shown in figure 3.3.

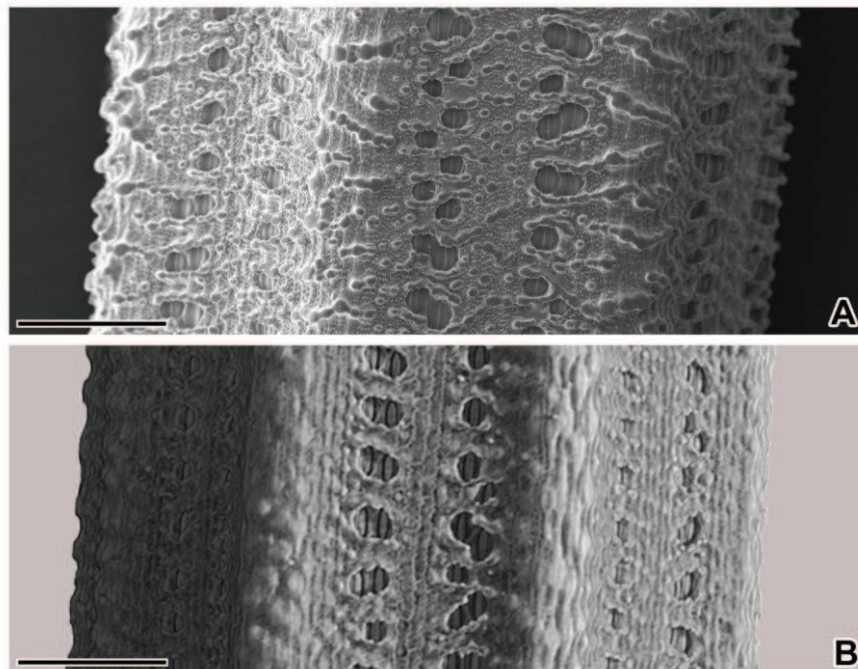


Figure 3.2: Comparison of SEM imaging (A) and 3D reconstruction of X-ray CT data (B) of the surface of an *Equisetum ramosissimum* stem. Scalebars: 300 μm .

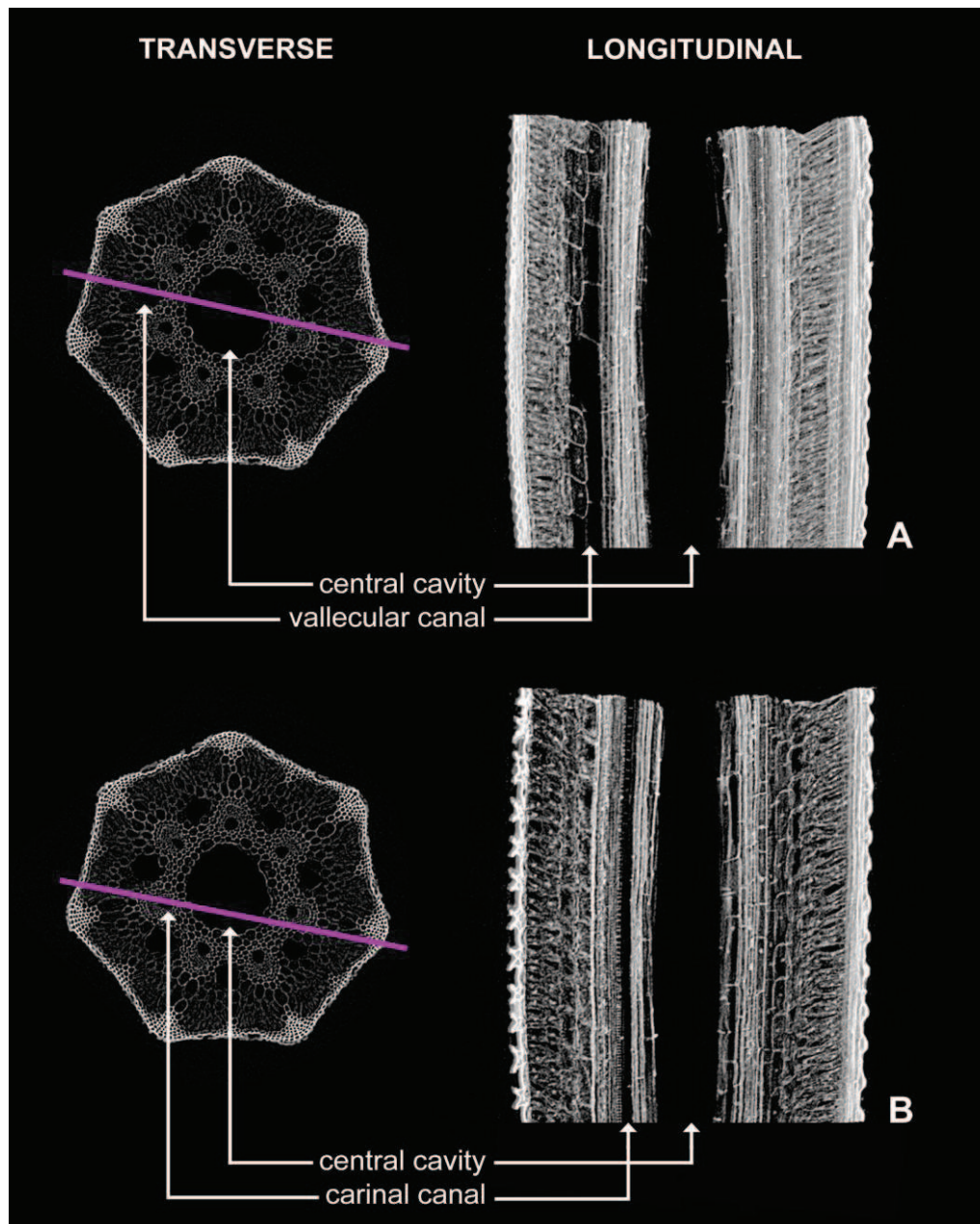


Figure 3.3: Comparison of transverse and longitudinal virtual sections through the same internodal stem segment of *Equisetum ramosissimum* showing the vallecular (A) and carinal (B) canals.

Apart from the advantages described above, X-ray VHRCT creates new possibilities to study plant structures. In the first place, the cutting plane can be modified until it matches an ideal or desired symmetry plane using powerful CT reconstructing software. Secondly, virtual transverse, longitudinal and oblique sections of one and the same structure are possible using the same software. Further progress in tomographic data collection using X-rays will establish its use as an important tool in many biological disciplines such as functional morphology, taxonomy, systematics and histology.

3.4 CONCLUSIONS

In conclusion, this method is particularly useful for studying fresh tissues and minimizes some of the disadvantages related to previously used techniques as (1) no cellular compression occurs as the tissues are completely fixed by critical point drying, (2) the contrast is enhanced due to heavy metal staining and critical point drying, and (3) embedding media are unnecessary and thus only the plant structures are imaged.

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Part 2

Aspleniaceae: root anatomical features

Histogeny, structure, composition and taxonomical significance of the sclerenchyma sheath in Aspleniaceae roots (Polypodiales)

This chapter is a modified version of:

Leroux O., Pizzi A., Leroux F., Masschaele B., Chabbert B., Knox P., Habrant A., Rigolet S., Bals S., Van Tendeloo G., Van Hoorebeke L., Viane R.L.L. Histogeny, structure, composition and taxonomical significance of the sclerenchyma sheath in Aspleniaceae roots (Polypodiales) (unpublished results).

Summary

Roots of Aspleniaceae are characterized by the presence of a sclerenchyma sheath. Detailed studies on the histogeny and structure of this sheath are needed to understand its taxonomical significance. Moreover, the cell wall composition of the sclereids and the origin of their typical colour are unknown. The sclerenchyma sheath was studied through light and electron microscopy as well as X-ray computed tomography. Cell wall composition was investigated by solid-state MAS+DEC ¹³C NMR, immunofluorescence labelling, and acetyl bromide and sugar analysis. The multilayered sclerenchyma sheath in *Asplenium*- and *Hymenasplenium* species originates from two early-separated cell-lineages and the inner cortex always remains single layered. Two monophyletic lineages were supported by, respectively, the mode of cell wall thickening, and the presence of only six cells in the inner cortex. Passage cells were found in all Asplenioid species. Our NMR analysis indicated the presence of high amounts of glucomannans and polyflavonoid tannins, and suggested the absence of lignins. Both sugar and acetylbromide analyses, and immunocytochemistry confirmed these results. We confirmed the taxonomical significance of two root anatomical characters: (1) the mode of thickening of sclereids, and (2) the number of inner cortex cells. We rejected an earlier hypothesis of parallel evolution of passage cells, and evidenced that the multilayered sclerenchyma sheath is not formed by periclinal divisions of the inner cortex. The sclereid cell walls are polylamellated non-lignified cell walls rich in glucomannans and highly polymerized polyflavonoid tannins and their yellowish-brown colour is most probably caused by oxidation of polyflavonoid tannins. The non-cellulosic polysaccharide composition of the sclereids resembles that of gymnosperm secondary cell walls, suggesting that mannan-containing polysaccharides are major hemicellulosic polysaccharides in (secondary) cell walls of early-diverging tracheophytes.

4.1 INTRODUCTION

The Aspleniaceae is one of the most species-rich leptosporangiate family, with over 720 species of terrestrial, epilithic or epiphytic ferns, distributed all over the world and even extending into Arctic and arid areas (Kramer and Viane, 1990). The number of genera recognized varies from one single genus *Asplenium* up to twenty. When more genera are recognized, the large genus *Asplenium* contains more than 90% of the species, with the satellite genera, such as *Ceterach*, *Diellia*, *Scolopendrium*, *Pleurosorus*, etc. (see e.g., Copeland, 1947; Pichi Sermolli, 1977) only having one or a limited number. All recent studies focusing on the molecular phylogenetics of Aspleniaceae (e.g., Murakami *et al.*, 1998, 1999; Vogel *et al.*, 1996; Gastony and Johnson, 2001; Schulze *et al.*, 2001; Pinter *et al.*, 2002; Van den heede *et al.*, 2003; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2008; Bellefroid *et al.*, 2010) confirm Kramer and Viane's (1990) concept that most of its small satellite genera should be merged into *Asplenium*. However, most authors now agree to recognize two genera, *Hymenasplenium* and *Asplenium*, within this large and diverse family. The analyses of Van den heede *et al.*, (2003) and Schneider *et al.* (2004), based on *rbcL* and *trnL-F* spacer sequences, are currently the most exhaustive studies based on molecular data. Additional molecular trees, recently published by Schuettpelz and Pryer (2008), Bellefroid *et al.* (2010), and Leroux *et al.* (2011), only show minor topological differences between the larger clusters in comparison to those in the earlier trees (Van den heede *et al.*, 2003; Schneider *et al.*, (2004). Essentially, Kramer and Viane's concepts (1990), based on morphological, anatomical, and cytological characters, are supported by all recent molecular studies.

Fern root meristems have been studied extensively (e.g., Pringsheim, 1863; Nägeli and Leitgeb, 1868; de Bary, 1877; Bower, 1889; Rumpf, 1904; Cross, 1937; von Guttenberg, 1966; Gunning *et al.*, 1978; Barlow *et al.*, 1982; Freeberg and Gifford, 1984; Gifford, 1983; Gifford, 1985; Eastman and Peterson, 1985; Gifford, 1991; Gifford, 1993; Hou and Hill, 2002; Hou *et al.*, 2004) and these studies showed that most ferns are characterized by the presence of a single root apical cell (RAC). In contrast, Osmundaceae (Guttenberg 1966; Freeberg & Gifford, 1984), as well as Marattiaceae (Koch, 1895; von Guttenberg, 1966) were reported to have several apical cells, especially in well established roots. Roots of species belonging to the Ophioglossaceae, the single other extant family within the eusporangiate ferns s.s., all display a single apical cell (von Guttenberg 1966; Gifford, 1983). Although molecular phylogenetic studies showed that *Equisetum* nests within a monophyletic lineage including the eusporangiate and leptosporangiate ferns (Pryer *et al.*, 2001; Pryer *et al.*, 2004; Schneider *et al.*, 2004; Smith *et al.*, 2006), the ontogeny of the root apical meristem, also consisting of a single tetrahedral apical cell, is atypical as the

endodermis and pericycle share a common precursor cell (Nägeli and Leitgeb, 1868; Gifford, 1993).

Derivations of the three proximal faces of the RAC have been referred to as merophytes by Douin in 1923 (Gunning *et al.*, 1978). By studying proximal merophytes of different relative ages, i.e. at different distances from the RAC, it is possible to study the development of tissues as cell divisions in the proximal merophytes occur in a strict sequence (de Bary, 1877; von Guttenberg 1966; Hou & Hill, 2004). Gifford (1991) presented the ontogeny of merophytes in roots of *Asplenium bulbiferum*, but only up to early stages of maturation.

The cortex of Aspleniaceae roots consists of thin-walled parenchyma cells and a clearly distinguishable sheath of mechanical tissue (Ogura, 1972; Schneider, 1996). This characteristic sclerenchymatous zone, which occurs in roots of many fern species, has been referred to as a 'gaine protectrice' (Van Tieghem, 1871), 'Stützscheide' or 'Sklerenchymmassen' (Russow, 1872), 'Stereome' or 'Stereomscheiden' (De Bary, 1877), 'anneau scléreux' (Poirault, 1893), 'mechanische Scheide' (Rumpf, 1904), 'supporting sheath' (Ogura, 1972), 'sclerenchyma sheath' (Chapple & Peterson, 1987) and 'sclerenchymatous inner cortex' (Schneider, 1997). In Aspleniaceae, Schneider (1997) also referred to the cells of the sclerenchymatous inner cortex cells as '*Asplenium*-sclereids', being sclereids with extremely thick inner tangential cell walls. As this type of sclereids also occurs in other distantly related fern genera such as *Adiantum*, *Austrogramme*, *Pleurosoriopsis*, and *Schizea* (Schneider, 1996), we prefer to use the terms 'sclereids' and 'sclerenchyma sheath' as proposed by Chapple & Peterson (1987). Studies of the root anatomy of Aspleniaceae were made by van Tieghem (1871), Rumpf (1904), Pelourde (1906), Tardieu-Blot (1932), and Schneider (1997). A typology of Aspleniaceae roots based on anatomical characters of their cortex was presented by Schneider (1997), highlighting their usefulness in taxonomical studies. Schneider *et al.* (2004) plotted root anatomical character states on a phylogenetic tree and recognized two synapomorphies: (1) unevenly thickened sclereids for the clade containing all *Asplenium* species, and (2) an inner cortex consisting of only six cells for a monophyletic clade comprised of *A. aethiopicum* and *A. affine* related species.

In this study we focused on the histogeny and structure of the sclerenchyma sheath in Aspleniaceae. We investigated the range of its variability in structure within and among species in relation to growth and development and we performed a detailed study of the cell wall composition and (ultra)structure of the root sclereids of *Asplenium elliotii*.

4.2 MATERIALS AND METHODS

4.2.1 Material

Fresh roots were obtained from our living collection kept at the Ghent Botanical Garden, Belgium. All species used in this study are listed in Table 4.1.

4.2.2 Light Microscopy

For hand-cut sections, roots were clamped in between two sheaths of Parafilm M (Pechiney Plastic Packaging Inc, Neenah, WI, USA) and sectioned using a single edged razor blade. For resin-sections, roots were fixed overnight in FAA (50% v/v ethanol, 5% v/v acetic acid and 5% v/v commercial formalin in distilled water), dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) following Leroux *et al.* (2007). Transverse sections of 4 µm were cut with a Microm HM360 microtome (Microm International GmbH, Walldorf, Germany) equipped with a holder for glass knives, dried on Vectabond-coated (Vector Laboratories, Burlingame, CA, USA) slides, and stained with an aqueous 0.05% w/v solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% w/v Na₂B₄O₇ and mounted in DePeX (Gurr, BDH Laboratory, UK). Phloroglucinol/HCl staining was performed on fresh hand-cut sections using 2% w/v phloroglucinol in 95% v/v ethanol for 5 min, and subsequently mounting in 33% v/v hydrochloric acid. Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

4.2.3 Transmission electron microscopy

Root cortex tissue of *A. eliottii* (Viane 7492) was dissected in order to produce blocks measuring approximately 5 mm length at all sides. They were fixed with 4% w/v formaldehyde and 2.5% v/v glutaraldehyde in a cacodylate buffer 0.1 M pH 6.9 for 24 h at 4 °C, washed in the same buffer and dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100% alcohol/LR White resin (1:1) at 4°C overnight, brought to 100% alcohol/ LR White (1:2) for 8 h (4°C), and transferred to 100% LR White resin and left overnight at 4°C. Polymerization was performed at 55°C for 16 h in gelatin capsules. 300-nm-thick sections were made using a Reichert ultracut S Ultramicrotome. Pioloform-coated 75-mesh copper grids were used to support the sections and a thin layer of amorphous carbon was evaporated to increase stability under the electron beam. The grids were examined with a FEI Tecnai Spirit Biotwin operating at 120kV and a 200 kV FEI Tecnai F20 equipped with a Fischione annular detector.

4.2.4 Scanning electron microscopy

Hand-cut sections of roots of *A. eliottii* (Viane 7492) were fixed in FAA, dehydrated in a graded ethanol series followed by a graded acetone series, and dried in a critical point dryer (Balzers CPD-030) using CO₂ as a transition fluid. Dried sections were mounted on clean aluminium stubs with double-sided adhesive graphite tabs. Mounted specimens were coated with gold (12-15 nm thick) using Balzers SPD-050 sputter coater. Sections were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

4.2.5 Immunohistochemistry and autofluorescence

Hand-cut sections were immunolabelled with the anti-homogalacturonan monoclonal antibodies JIM5 and JIM7 (Clausen *et al.*, 2003); anti-galactan antibody LM5 (Jones *et al.*, 1997), anti-arabinan antibody LM6 (Willats *et al.*, 1998), anti-xylan antibody LM11 (McCartney *et al.*, 2005), anti-xyloglucan antibody LM15 (Marcus *et al.*, 2008), and anti-mannan LM21 (Marcus *et al.*, 2010) as described in Leroux *et al.* (2009). After mounting, slides were viewed using a laser scanning confocal microscope (Nikon e-C1 mounted on a Nikon TE2000). Photos were taken at an X/Y resolution of 512x512 pixels, after exciting the staining signal at 543 or 488 nm using a HeNe and Argon laser respectively and detecting the fluorescence emission with either a 571 or 540 nm long pass filter.

4.2.6 X-ray Computed Tomography and drawings

Roots of *A. theciferum* (Bellefroid 238) and *A. eliottii* (Viane 7492), fixed in FAA and processed following Leroux *et al.* (2009), were scanned at the centre for computed tomography (UGCT) of Ghent University (Masschaele *et al.*, 2007). For the measurements we used an in-house scanner the main components of which are: an open type Hamamatsu nanofocus X-ray tube (L10711); a second sealed type Hamamatsu microfocus (L9181-02); a high resolution CCD camera with fiber optic taper from the company Photonic Science; a Varian amorphous silicon flat panel detector (Paxscan 2520 with CsI scintillator), and a sample manipulator which can move the samples with 5 degrees of freedom. The samples were scanned with the L9181 tube at 70 kV and 60 μ A in order to obtain high contrast and high spatial resolution. The samples were rotated from 0° to 360° in steps of 0.45°. The Varian detector was set to an exposure time of 400 ms and 6 frames were averaged per rotation step to increase the statistics of the images. The samples were magnified 42 times in order to reach a voxel size of 3 μ m in the CT images. The raw projection images were processed into CT slices with Octopus. Octopus is a user friendly, system independent, reconstruction package developed and commercialized by

Ghent University (www.inct.be). After the reconstruction we obtained over 800 cross sections through the sample volume. The software programme VGStudio Max (<http://www.volumegraphics.com>) was used to create the 3D renderings. Drawings were based on a selection of virtual cross sections and were made with CorelDRAW Graphics Suite 12.

4.2.7 ¹³C Nuclear magnetic resonance

Root sclerenchyma sheaths were isolated by dissecting mature roots of *A. elliotti* (Viane 7492). The obtained cell wall material was lyophilized with liquid nitrogen and subsequently pulverized in a shaker. They were analyzed by solid-state MAS+DEC (proton decoupling) ¹³C NMR. With the DEC technique the spectra are quantitative, contrary to what would be obtained by CP-MAS. Spectra were obtained on a Bruker MSL 300 FT-NMR spectrometer at a frequency of 75.47 MHz and at sample spin of 4.0 kHz. The impulse duration at 90° was 4.2 ms, contact time was 1 ms, number of transients was about 1000, and the decoupling field was 59.5 kHz, d1=60s. Chemical shifts were determined relative to tetramethyl silane (TMS) used as control. The spectra were accurate to 1ppm. The spectra were run with suppression of spinning side bands.

4.2.8 Samples for lignin determination and sugar analysis

Prior to chemical analysis, isolated sclereids of *A. elliotti* (Viabe 7492) were ground with a ball crusher (Retsh, MM 2000) and extracted sequentially at 40 °C by ethanol 80 %, acetone 70 %, and water.

4.2.9 Acetyl bromide analysis

Tentative determination of lignin was carried out by recording UV spectra after acetyl bromide/acetic acid treatment (Iiyama and Wallis, 1990) allowing dissolution of the cell wall residue. Briefly, 10 mg of samples were submitted to acetyl bromide/acetic acid treatment for 30 minutes at 70 °C prior to spectrophotometric analysis.

4.2.10 Sugar analysis

Neutral and acid sugar analysis was performed by acid hydrolysis of 10 mg cell wall residue using 12 M H₂SO₄ (2 h at room temperature) followed by 1.5 M H₂SO₄ for 2 h at 100 °C. Acid soluble samples were subsequently filtered and injected into a CarboPac PA1 anion exchange column (4 × 250 mm, Dionex). Detection was performed by pulsed amperometry (PAD 2, Dionex). A postcolumn addition of 300 mM NaOH was used (Beaugrand *et al.*, 2004).

Table 4.1: List of specimens and observations based on resin and hand-cut sections.

Species	Voucher number / Literature reference	Locality	Number of layers of inner and outer cortex sclereids	Number of inner cortex sclereids:	Mode of thickening of sclereid cell wall: thickening mainly restricted to the inner tangential cell wall (A) or uniformly thickened (B)	Passage zones: present (+) or absent (-)
<i>A. actiniopteroides</i>	Viane 7583	Tanzania	1 + 1	6	A	+
<i>A. aethiopicum</i> ssp. <i>tripinnatum</i>	Viane 7377	Kenya	1 + 0	6	A	+
<i>A. aethiopicum</i>	Viane 10564	Portugal	1 + 0	6	A	+
<i>A. aethiopicum</i> ssp. <i>aethiopicum</i>	Viane 7412	Kenya	1 + 0	6	A	+
<i>A. aethiopicum</i> ssp. <i>aethiopicum</i>	Viane 11230	Uganda	1 + 1	6	A	+
<i>A. affine</i>	Viane 8227	Réunion	1 + 0	6	A	+
<i>A. amboinense</i>	Schneider <i>et al.</i> (1997)*	NA	1 + ≥ 1	>6	A	NA
<i>A. angustum</i>	Schneider <i>et al.</i> (1997)*	NA	1 + ≥ 2	>6	A	NA
<i>A. alatum</i>	Viane 10231	Venezuela	1 + 3	>6	A	+
<i>A. anisophyllum</i>	Viane 6402b	Rep. South Africa	1 + 4	>6	A	+
<i>A. anisophyllum</i>	Viane 8775	Zimbabwe	1 + 4	>6	A	+
<i>A. auriculatum</i>	Viane 10602	Puerto Rico	1 + 2	>6	A	+
<i>A. auritum</i>	Viane 10368	Venezuela	1 + 3	>6	A	+
<i>A. auritum</i>	Viane 10628	Puerto Rico	1 + 1	>6	A	+
<i>A. bulbiferum</i>	BGGU s.n.	BGGU, location unknown	1 + 1	>6	A	+
<i>A. centrafricanum</i>	Bellefroid 393	Uganda	1 + 3	>6	A	+
<i>A. centrafricanum</i>	Bellefroid 392	Uganda	1 + 2	>6	A	+
<i>A. ceterach</i>	Van den Heede 250b	Cyprus	1 + 3	>6	A	+
<i>A. christii</i>	Viane 8792	Zimbabwe	1 + 1	>6	A	+
<i>A. compressum</i>	Viane 8851	St. Helena	1 + 2	>6	A	+
<i>A. cordatum</i>	Viane 11567B	Rep. South Africa	1 + 2	>6	A	+
<i>A. cordatum</i>	Viane 11569	Rep. South Africa	1 + 1	>6	A	+
<i>A. crinicaule</i>	Viane 11304	China	1 + 1	6	A	+
<i>A. currorii</i>	BGGU 792824	Ivory coast	1 + 2	>6	A	+

<i>A. cuspidatum</i>	Viane 10108	Venezuela	1 + 0	>6	A	+
<i>A. daucifolium</i> ssp. <i>lineatum</i>	Viane 8220	Réunion	1 + 2	>6	A	+
<i>A. daucifolium</i> ssp. <i>viviparum</i>	Viane 8445	Réunion	1 + 3	>6	A	+
<i>A. decompositum</i>	Viane 7688	Tanzania	1 + 1	6	A	+
<i>A. dregeanum</i>	Viane 11199	Uganda	1 + 2	>6	A	+
<i>A. elliotii</i>	Viane 7492	Kenya	1 + 5	>6	A	+
<i>A. elliotii</i>	Viane 7685	Tanzania	1 + 3	>6	A	+
<i>A. erectum</i>	Viane 11247	Uganda	1 + 1	>6	A	+
<i>A. exiguum</i>	Viane 11102	Mexico	1 + 2	>6	A	+
<i>A. finlaysonianum</i>	Viane 9708	Myanmar	1 + 1	>6	A	+
<i>A. friesiorum</i>	Viane 11248	Uganda	1 + 1	6	A	+
<i>A. friesiorum</i>	Viane 8752	Zimbabwe	1 + 0	6	A	+
<i>A. gemmiferum</i>	Viane 11117	Uganda	1 + 1	>6	A	+
<i>A. hastatum</i>	Viane 10182c	Venezuela	1 + 0	>6	A	+
<i>A. hemionitis</i>	BGGU s.n.	BGGU, location unknown	1 + 0	>6	A	+
<i>A. juglandifolium</i>	Viane 10667	Puerto Rico	1 + 0	>6	A	+
<i>A. kukkonenii</i>	Viane 10886	China	1 + 0	>6	A	+
<i>A. loxoscaphoides</i>	Viane 7549	Tanzania	1 + 0	>6	A	+
<i>A. lunulatum</i>	Viane 6558	Rep. South Africa	1 + 2	>6	A	+
<i>A. majus</i>	Viane 7427	Kenya	1 + 0	6	A	+
<i>A. marinum</i>	Viane 10568	Portugal, Madeira	1 + 3	>6	A	+
<i>A. monanthes</i>	Viane 11106	Mexico	1 + 1	>6	A	+
<i>A. myriophyllum</i>	Viane 10147	Venezuela	1 + 5	>6	A	+
<i>A. nidus</i> s.l.	Viane 9629	Myanmar	1 + 2	>6	A	+
<i>A. nitens</i>	Viane 8203	Réunion	1 + 1	>6	A	+
<i>A. obtusatum</i>	Schneider <i>et al.</i> (1997)*	NA	1 + ≥ 1	>6	A	NA
<i>A. onopteris</i>	Viane 8078	Spain	1 + 0	6	A	+
<i>A. pekinense</i>	Viane 10031	China	1 + 1	>6	A	+
<i>A. petiolulatum</i>	Viane 8423	Réunion	1 + 1	>6	A	+
<i>A. polyodon</i>	Viane 11217	Uganda	1 + 1	>6	A	+
<i>A. preussii</i>	Viane 8798	Zimbabwe	1 + 1	>6	A	+
<i>A. preussii</i>	Viane 11193	Uganda	1 + 0	>6	A	+
<i>A. prolongatum</i>	Viane 11314	China	1 + 0	>6	A	+

<i>A. pteropus</i>	Viane 10645	Puerto Rico	1 + 1	>6	A	+
<i>A. radicans</i>	Viane 10639	Puerto Rico	1 + 1	>6	A	+
<i>A. ruta-muraria</i>	Viane 10040	France	1 + 0	>6	A	+
<i>A. rutifolium</i>	Bellefroid 245	Zimbabwe	1 + 2	>6	A	+
<i>A. rutifolium</i>	Bellefroid 249	Zimbabwe	1 + 1	>6	A	+
<i>A. sandersonii</i>	Viane 7719A	Tanzania	1 + 1	>6	A	+
<i>A. scalare</i>	Schneider <i>et al.</i> (1997)*	NA	1 + ≥ 1	>6	A	+
<i>A. scolopendrium</i>	Viane 10971	Belgium	1 + 1	>6	A	+
<i>A. septentrionale</i>	Viane 9578	France	1 + 1	>6	A	+
<i>A. serratum</i>	Viane 10607	Puerto Rico	1 + 3	>6	A	+
<i>A. smedsii</i>	Viane 7195	Ethiopia	1 + 2	>6	A	+
<i>A. smedsii</i>	Viane 7707	Tanzania	1 + 1	>6	A	+
<i>A. squamulatum</i>	Njo s.n.	Indonesia	1 + 2	>6	A	+
<i>A. sublaserpitifolium</i>	Viane 11266	China	1 + 1	6	A	+
<i>A. tenerum</i>	Viane 9043	Indonesia	1 + 1	>6	A	+
<i>A. tenuicaule</i>	Viane 10888	China	1 + 0	>6	A	+
<i>A. theciferum</i>	Bellefroid 238	Zimbabwe	1 + 2	>6	A	+
<i>A. theciferum</i>	Bellefroid 247	Zimbabwe	1 + 1	>6	A	+
<i>A. theciferum</i>	Viane 10210	Venezuela	1 + 2	>6	A	+
<i>A. trichomanesssp. trichomanes</i>	Viane 6351	Italy	1 + 1	>6	A	+
<i>A. trichomanes ssp. quadrivalens</i>	Viane 10519	Russian Fed.	1 + 2	>6	A	+
<i>A. varians ssp. varians</i>	Viane 10733	China	1 + 0	>6	A	+
<i>A. varians ssp. fimbriatum</i>	Viane 11521	Rep. South Africa	1 + 1	>6	A	+
<i>A. volkensii</i>	Viane 11124	Uganda	1 + 0	6	A	+
<i>A. volkensii</i>	Viane 7313	Kenya	1 + 3	6	A	+
<i>A. vulcanicum</i>	Njo 35	Indonesia	2	>6	A	+
<i>A. yoshinagae</i>	Viane 9395	China	1	>6	A	+
<i>H. excisum</i>	Viane 11263	China	1	>6	B	+
<i>H. obscurum</i>	Viane 11286	China	1	>6	B	+
<i>H. unilaterale</i>	Viane 8344	Réunion	2	>6	B	+

* Character states taken from literature: missing data indicated with NA; BGGU: Botanical Garden Ghent University.

4.3 RESULTS

Histogeny, structure and cell wall composition

In general characteristics, root structure of Aspleniaceae is similar to that of most other leptosporangiate ferns (Ogura, 1972). The outermost limit consists of a one cell layer thick rhizodermis (Fig. 4.1). The cortex is characterized by three distinct layers: an outer parenchymatous layer, a sclerenchyma sheath consisting of sclereids, often interrupted at the protoxylem poles, and a single-layered endodermis (Fig. 4.1). The central cylinder consists of a pericycle surrounding the diarch radial vascular bundle. The xylem is exarch and composed of a small number of tracheids and is flanked on both sides by phloem consisting of sieve cells and phloem parenchyma.

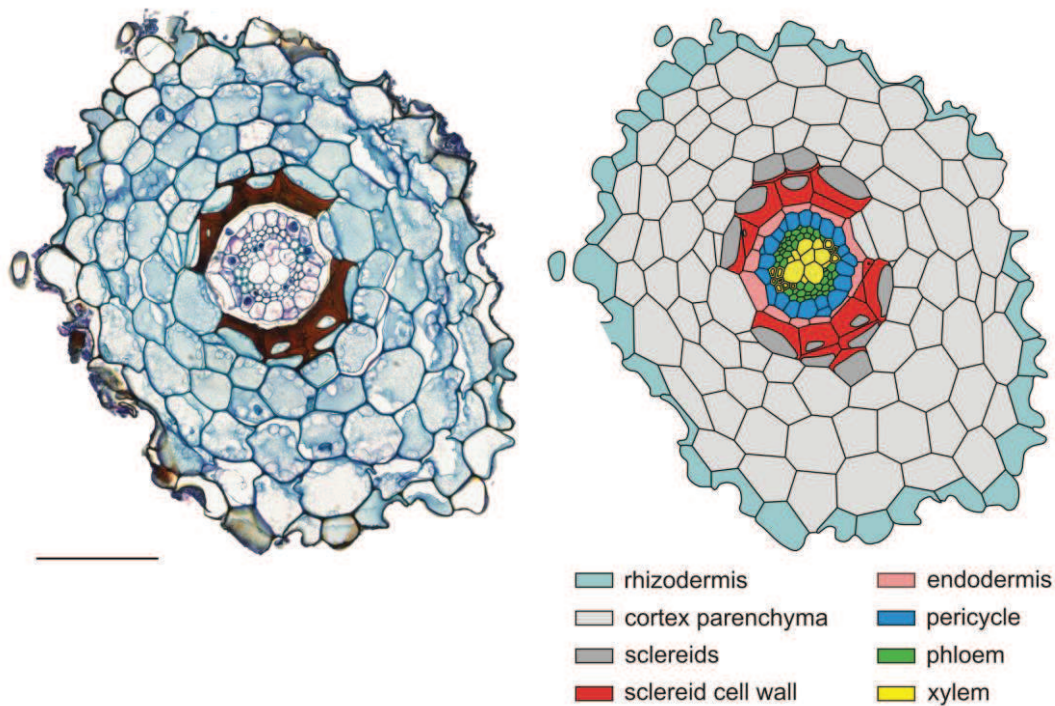


Figure 4.1: Transverse section of the root of *Asplenium alatum* and corresponding schematic drawing with colour indication of tissues. scalebar: 100 μm .

The root apical cell (RAC) and early cell divisions

We studied the root ontogeny through serial transverse, longitudinal and oblique sections of root tips of *A. aethiopicum* (Viane 7412, Viane 10564), *A. elliotii* (Viane 7492, Viane 7685), *A. daucifolium* (Viane 8220), *A. theciferum* (Bellefroid 238, Bellefroid 247, Viane 10210), *A. marinum* (Viane 10568), *A. scolopendrium* (Viane 10971), and *H. obscurum* (Viane 11286). These species were chosen as they represent the major clades within Aspleniaceae recognized by Schneider *et al.* (2004).

The RAC of *Asplenium* and *Hymenasplenium* roots is a tetrahedral cell (Fig. 4.2A) with four division faces. Cells directly cut off from the RAC are referred to as merophytes (Fig. 4.2A, inset), which, altogether, contribute to the construction of the main body of the root. Root cap initials are generated from the distal face of the RAC, but are not discussed here. Two types of divisions are recognized in developing proximal merophytes (Gunning *et al.*, 1978): formative divisions and proliferative divisions. Formative divisions are longitudinal (either radially longitudinal or periclinally) resulting in daughter cells which will further develop into different tissue-types. Proliferative divisions, on the other hand, increase the number of cells within the same tissue-type. Radial proliferative divisions increase the number of cells in a single layer, whereas periclinal proliferative divisions add additional layers in each merophyte. Transverse divisions are proliferative and result in the formation of files of cells.

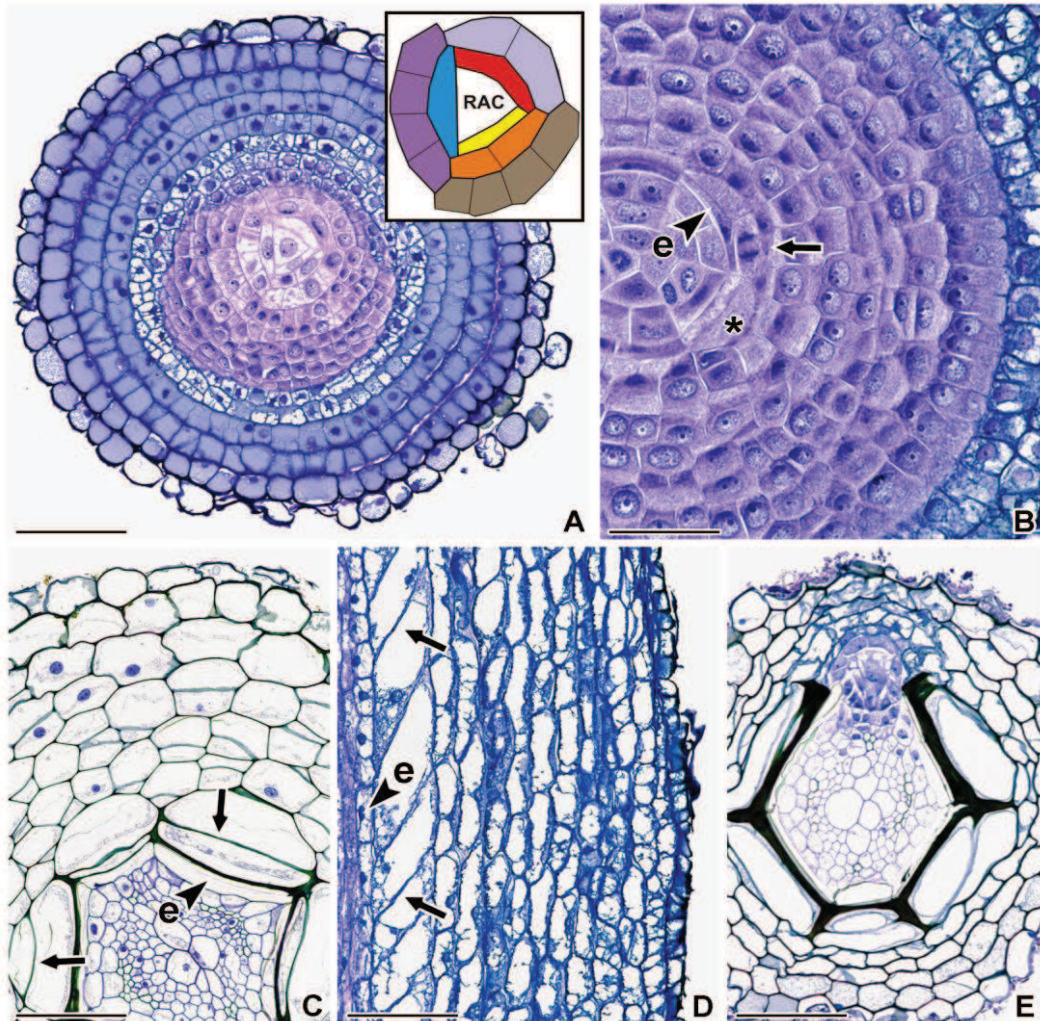


Figure 4.2: Transverse (A, B, C, E) and longitudinal (D) section of roots of *Asplenium eliottii* (Viane 7492) (A, B) and *Asplenium aethiopicum* (Viane 10564) (C, D, E). **A.** The single apical cell surrounded by merophytes. Inset: schematical drawing showing seven merophytes, each in a different colour. **B.** Evidence of radially dividing inner cortex precursor (arrow). Inner cortex cell opposite protoxylem (indicated with an asterisk) does not undergo radial divisions. **C.** Section showing four out of six inner cortex cells surrounding the endodermis (e) and central stele. Oblique transverse cell walls between two superimposed inner cortex cells indicated with arrows. **D.** Oblique transverse cell walls (arrows) of the inner cortex cells adjacent to the endodermis in a young root (e). **E.** Evidence of developing lateral root. Abbreviations: e, endodermis. Scalebars: A, 100 μm ; B, 50 μm ; C, 200 μm ; D, E: 150 μm .

The early sequence of divisions of the proximal merophytes in the species we studied corresponded with those reported for *Asplenium bulbiferum* by Gifford (1992) and are summarized in Figure 4.3. The first division of a proximally produced merophyte is periclinal and asymmetrical, resulting in the formation of a large inner and small outer cell. The outer cell divides radially longitudinal and periclinally and ultimately produces precursors for the rhizodermis and outer cortex. The inner cell first undergoes a radial longitudinal division forming two daughter cells, referred to as 'sextants', as each of them occupy approximately one-sixth of the root body seen in transverse view. Subsequent periclinal divisions in each sextant establish precursors for the inner cortex, the endodermis, the pericycle and the vascular tissue. As shown in Figure 4.4, the six sextants (two in each of the three proximal merophytes) can be recognized, even at a considerable distance from the RAC. As Gifford (1992) only provided data up to the formation of early precursors of the root body, further details were required to fully assess the origin of the sclerenchyma sheath. These studies revealed novel findings which are discussed in the next sections. The formative divisions of the vascular tissue were not studied (Fig. 4.3).

Origin of the sclerenchyma sheath

A periclinal division of a common precursor cell originating from the inner cell produces the inner cortex and the endodermis precursors (Fig. 4.3), which then only divide radially and transversely in the roots of most species. Notwithstanding the fact that - when observing transverse sections of roots - some inner cortex cells look as if they have divided periclinally (Fig. 4.2C), we did not observe periclinal divisions of inner cortex cells in any of our serial sections. Moreover, such divisions are impossible once inner cortex cells start to sclerify, which happens very close to the apex. This contradiction is clarified through examination of longitudinal sections of root tips. Transverse walls of inner cortex cells of both young and mature roots are oblique, with an angle ranging from 20° to 35° in relation to the longitudinal axis of the root (Fig. 4.2D). Consequently, in transverse sections, the impression that the inner cortex cells have divided periclinally to form a 2-celled layer is caused by the fact that the plane of cutting transverses two superimposed cells and their strongly oblique transverse wall. Consequently, the inner cortex is always single layered, and in most cases, the boundary between the outer- and inner cortex is clearly visible. For instance, in roots of *Asplenium aethiopicum* and related species, the six cells of the inner cortex are easily distinguishable from the smaller sclerified outer cortex cells (Fig. 4.11D).

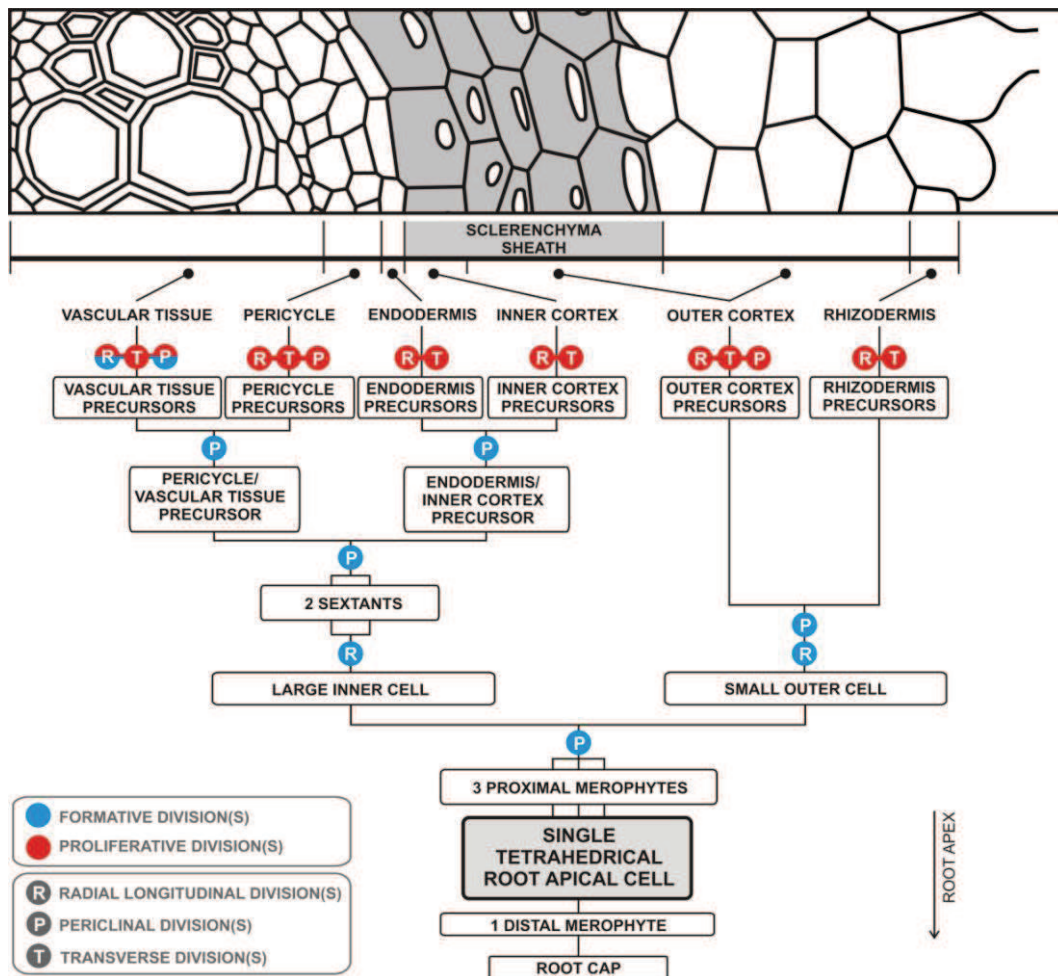


Figure 4.3: Cell lineage flow chart showing the origin and development of tissues in a merophyte and contribution of each of the cell lines to the tissues in a mature root with a multilayered sclerenchyma sheath. The three proximal merophytes follow the same sequence of divisions, and therefore only one is shown (similar for the two sextants).

Number of sclereids in the inner cortex

The number of inner cortex cells, as seen in transverse sections, depends on the amount of additional radial divisions (Fig. 4.4). After periclinal division of the endodermis/inner cortex precursor cell, six inner cortex precursors are established, one in each sextant (Fig. 4.4A). In *A. aethiopicum* and related species, no additional radial divisions occur and six large inner cortex precursors remain (Fig. 4.4A, Table 4.1) and these only undergo transverse divisions. In other species such as *A. eliottii* (Fig. 4.2B) and *A. marinum*, radial divisions give rise to additional inner cortex cells, and sometimes up to 30 inner cortex

cells can be distinguished (Fig. 4.4B, Table 4.1). However, these radial divisions do not occur in all sextants. The inner cortex precursor cells of the two sextants comprising the protoxylem do not undergo radial divisions as in the remaining four sextants (Fig. 4.4B). It should be noted that, in species with only six inner cortex cells, the inner cortex cells opposite both protoxylem poles are the smallest (Fig. 4.4A). These cells often remain unthickened and contribute to the establishment of passage zones which are described in more detail in the next section. Further differences in structure and its taxonomical implications are discussed further below.

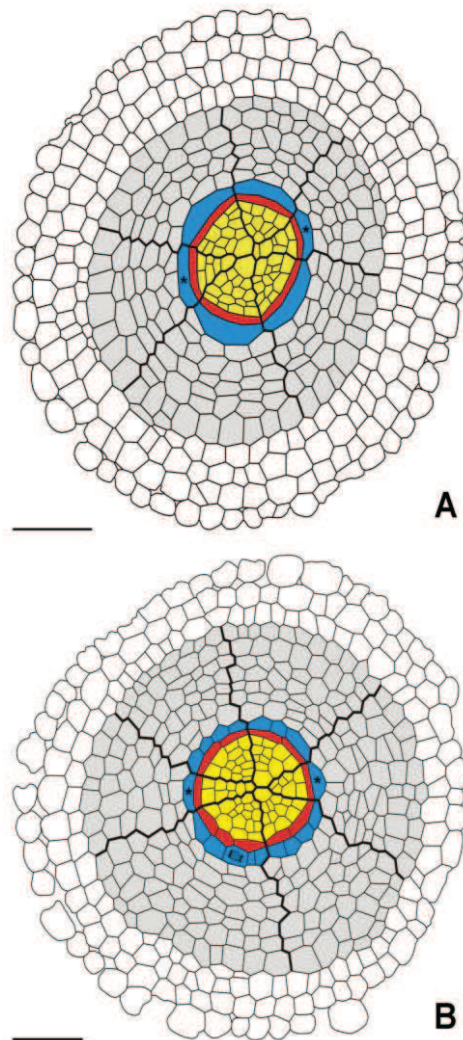


Figure 4.4: Schematic representation of transverse sections of root tips showing early developmental stages of the root. A. Six inner cortex and six endodermal cells in *A. aethiopicum*. **B.** Radial divisions in the six inner cortex initials produced 18 inner cortex and 11 endodermis precursors in *A. marinum*. White: rootcap; grey: outer cortex and protoderm; blue: inner cortex; red: endodermis; yellow: vascular stele. The sextants (two for each merophyte) are separated by bold lines. Inner cortex cells facing protoxylem poles indicated with asterisk. Scalebars: 100 μm.

Distribution of passage zones

The sclerenchyma sheath is often interrupted opposite one or both of the protoxylem poles. Nano X-ray computed tomography was used to generate three-dimensional views of the distribution of these interruptions in the roots of *A. theciferum* (Bellefroid 238) and *A. elliottii* (Viane 7492). Both species generally gave the same results. Figure 4.5A is a virtual transverse section of a root of *A. theciferum* with a sclerenchyma sheath consisting of three layers of sclereids. Although individual sclereids cannot be distinguished, their position is suggested by their remaining cell lumen. Differentiation of the metaxylem was not fully completed as the central tracheids did not develop secondary cell walls. A virtual radial section through the protoxylem poles of the same root is shown in figure 4.5B. Opposite these poles, the sclerenchyma sheath is locally interrupted by several parenchymatous cells. The thickness of the cell walls of the sclereids surrounding these parenchymatous cells gradually increases, causing elongate narrow serial depressions in the sclerenchyma sheath opposite the protoxylem. Figure 4.5C depicts a virtual tangential section through a depression zone showing a stack of four unthickened parenchymatous cells of the inner cortex. Due to the specific distribution of these passage zones throughout the root, they are not visible in each transverse section. Extensive screening within Aspleniaceae showed that they were present in all species studied (Table 4.1). In some sections, they are located opposite one or both of the protoxylem poles (Fig. 4.1), in others they are absent.

During our study we frequently observed evidence of lateral root initiation in the endodermis (data not shown) and of developing lateral roots growing through the passage zones of the sclerenchyma sheath (Fig. 4.2E). In each of these cases, lateral root initiation preceded sclerification of the inner cortex.

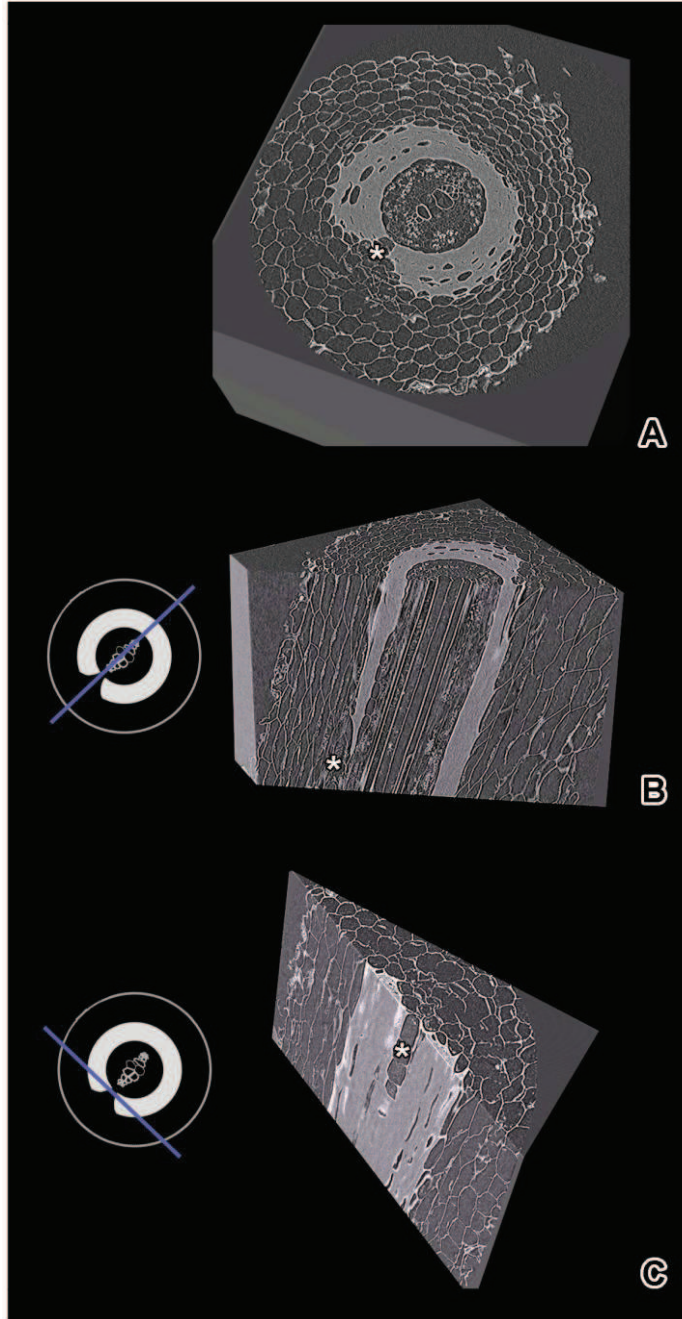


Figure 4.5: Virtual sections of three dimensional reconstructions of X-ray computed tomography data obtained after scanning a root of *A. theciferum*. A.

Transverse section showing a multilayered sclerenchyma sheath interrupted by a passage zone (*). Note that separate sclereids are not distinguishable, but their remaining lumen indicates their position. **B.** Radial sections (through protoxylem poles) showing that the passage zone (*) consists of unsclerified cells. **C.** Tangential section through a passage zone of the sclerenchyma sheath showing a stack of four parenchymatous cells.

Ultrastructure and composition of the sclereid cell wall of A. elliotii

The sclerenchyma sheath consists of hard sclereids with extremely thickened, yellowish-brown cell walls. In contrast to the sclereid cell walls in *Hymenasplenium*, which are evenly thickened, *Asplenium* sclereid walls are characterized by extremely thickened inner tangential secondary cell walls which, at maturity, occupy at least 90% of the total cell volume, leaving only a small eccentric cell lumen (Fig. 4.6). The ultrastructure of the sclereid cell wall of *A. elliotii* was studied using annular dark field scanning transmission electron microscopy (ADF-STEM). In a first attempt, 70 nm thick sections were made for observation with conventional bright field transmission electron microscopy (BF-TEM), but the extremely hard sclereid cell walls were difficult to section as they were poorly infiltrated with embedding medium, probably due to the presence of highly polymerized phenolic compounds (see further below). To avoid sectioning artefacts, 300 nm thick sections were made and these – devoid of contrast-enhancing staining – were investigated with conventional bright-field transmission electron microscopy (BF-TEM). The section thickness and compact nature of the sclereid cell wall resulted in severe aberrations due to the inelastic scattering of the electrons within the section. Hence, imaging at higher magnifications resulted in blurred images. To enable the fine-structural analysis of such sections an alternative technique called annular dark field scanning transmission electron microscopy (ADF-STEM) was employed. By using ADF-STEM, a small electron probe is scanned across the sample and only the highly scattered electrons are used to form an image. The resolution, when imaging thick sections, is not degraded by chromatic aberrations as no post-specimen imaging-forming lenses are used. Moreover, the intensity in ADF-STEM images can be directly related to the local mass in the section. As no heavy elements are present in the cell wall polymers and sections have a uniform thickness, brighter regions in the image will correspond to a higher mass density. BF-TEM showed that the secondary cell walls are polylamellated with up to 30 lamellae (Fig. 4.6A). Higher magnifications using ADF-STEM indicated that this clear-cut layering is caused by the alternation of lamellae having a predominantly transverse microfibril orientation with those having a predominantly longitudinal orientation (Fig. 4.6C). Scanning electron microscopy of the surface of a razor-blade-sectioned sclereid cell wall (Fig. 4.6C, inset) showed that the lamellae with a predominantly longitudinal orientation were damaged, whereas the others remained smooth. The lamellae also vary in composition as both binding of the anti-mannan LM21-antibody (Fig. 4.6C) and red autofluorescence – probably caused by phenolic compounds – (Fig. 4.6D) showed different intensity levels of fluorescence of the lamellae in the sclereid cell walls. We did

not achieve labelling of sclereid cell walls with any of antibodies directed against xylans (LM11), xyloglucan (LM15) and pectins (JIM5, JIM7, LM5, LM6).

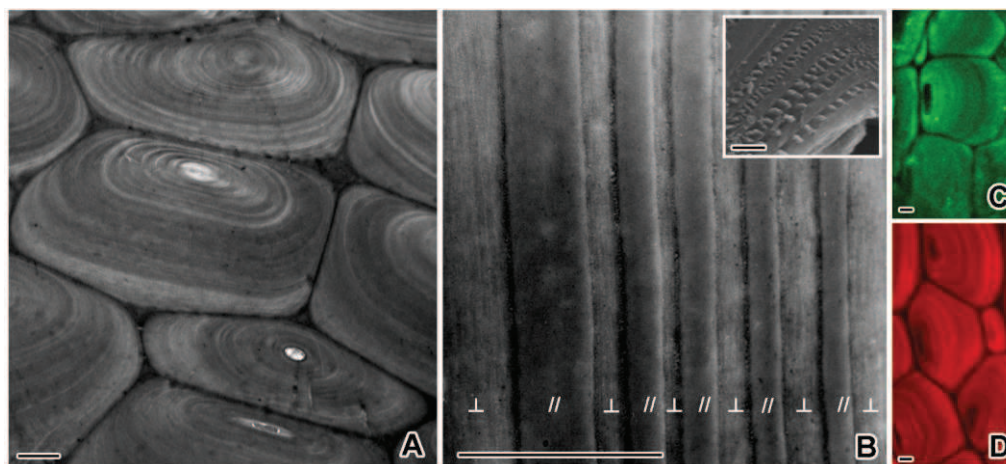


Figure 4.6: Bright Field Transmission Electron Microscopy (BF-TEM) (A) and Annular Dark Field Scanning Transmission Electron Microscopy (ADF-STEM) (B) of a 300 nm unstained section through the sclerenchyma sheath of *A. elliotii* and (immuno)fluorescent observations (C, D). **A.** Image showing the extremely thickened inner tangential secondary cell walls of sclereids. This cell wall occupies at least 90% of the cell volume and leaves only a small cell lumen. **B.** detail of the polylamellated secondary cell wall of a sclereid. The fibrils in the lamellae indicated with '⊥' are orientated perpendicularly to the long axis of the root, '/' indicates lamellae with microfibrils orientated at a small angle to the root axis. Inset shows cutting surface of a razor-blade-sectioned sclereid cell wall. Lamellae with microfibrils orientated at a small angle to the root axis are damaged. **C.** Binding of LM21 anti-mannan to the cell walls of the sclereids. Note that some lamellae showed weaker binding. **D.** Heterogeneity in intensity of red autofluorescence in the polylamellated sclereid cell walls. Scalebars: 5 μ m.

Solid-state CP-MAS ^{13}C NMR was used to analyse powdered sclereid cell walls of *A. elliotii*. This species was chosen as its roots have thick multilayered sclerenchyma sheaths and because it is gemmiferous and hence easily cultivable in large numbers to allow isolation of sufficient cell wall material. The obtained spectrum (Fig. 4.7) is dominated by the pattern characteristic of polymeric carbohydrates represented by the bands at 173.7 ppm (C=O group of acetyls on hemicelluloses), 104.9 ppm (C1), 89.6 ppm (C4 crystalline cellulose), 83.6 ppm (C4 amorphous carbohydrates mainly polymeric hemicelluloses), 75.3 ppm (C2 of all carbohydrates), and the shoulders at 73 ppm (C3,C5 of all carbohydrates), 65.1 ppm (C6 of crystalline cellulose), 62.2 ppm (C6 amorphous hemicelluloses), 58.7 ppm (C6 of amorphous cellulose), and 18-21 ppm ($-\text{CH}_3$ signal of acetyl groups on hemicelluloses). Based on the spectra of the polymeric carbohydrates it

is evident that hemicelluloses dominate by far. What appears as crystalline cellulose is rather limited and could either be crystalline cellulose or glucose enchainment in hemicelluloses if these are glucomannans. The sample contains phenolic materials. The flavonoid tannin's free C4 is almost absent indicating that the C4 site of the lower terminal flavonoid unit of the tannins present is either in the form of a carbonyl group or is linked to a sugar. Both are likely but the first is the most likely, as there is a broad peak at 164.9 ppm indicating a carbonyl group, the shift of which is too low to belong to a quinone derived from any oxidation of the phenolic hydroxyl-groups. As to the nature of the phenolic material this could either belong to lignin or to polyflavonoid tannins (condensed tannins). If lignins are present this is only in very small amounts. The broad peak at 164 ppm indicates that the signals of C5, C7 and C9 of a flavonoid tannin – in particular the shoulders at 158-162 ppm – are superimposed with the carbonyl group peak. The band at 145.7 ppm is characteristic of the C3' and C4' of a flavonoid tannin catecholic or pyrogallolic B-rings. Characteristic of polyflavonoid tannins are also the C1 bands at 129 and 131.4 ppm. The broad band centering at 117.5 ppm is the superposition of the signals of C6' (120 ppm), and C5' and C2' of (115-117 ppm) of the B-rings of the flavonoids. The relative importance of these peaks in the spectra indicates that the tannin B-ring is predominantly catecholic. At 104.97 (105 ppm) we find the characteristic interflavonoid bond which suggests that the substituted C4 of the flavonoid unit is linked to another flavonoid unit. This peak is prominent, indicating that the tannins have a relatively high level of polymerisation and this might explain the observed lack of solubility of some components in the sclereid cell walls. The fact that the 105 ppm band predominates while a band at 110 ppm does not appear could indicate that the main linkage between the flavonoid units in the tannin is predominantly C4 to C6 rather than C4 to C8. The series of shoulders between 95 and 101 ppm are those of the flavonoids C10, and free C6 and C8. Some of the shoulders at 95 to 101 ppm could also belong to lignin, and so could the two bands at 131 and 129 ppm. However, these two bands could be the superposition of tannin and lignin, and judging from their intensity relative to the other bands of phenolic materials, it is evident that if lignin occurs its proportion is very small, in fact the smallest of all the materials present. The only other peak that could indicate traces of lignin is the small peak at 52 ppm, simply because this is not assigned.

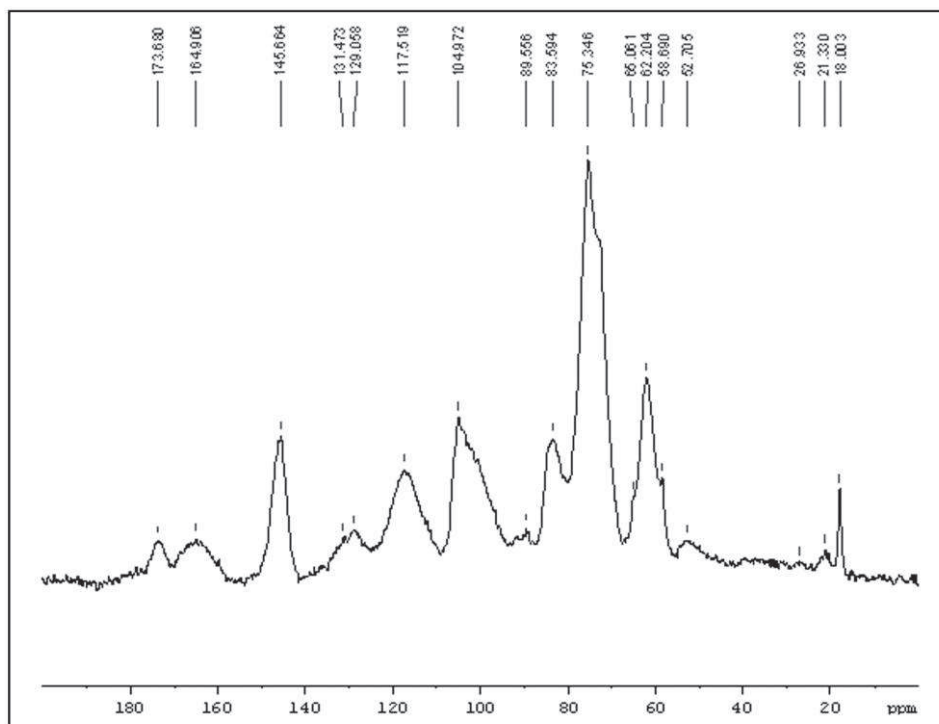


Figure 4.7: Solid-state MAS+DEC ^{13}C -NMR of root sclereids of *A. elliotii*. The spectrum is dominated by the pattern characteristic of polymeric carbohydrates (glucomannans) and highly polymerized polyflavonoid tannins.

The lignin content was further assessed by acetylbromide treatment. The UV spectra of acetylbromide-treated cell wall residue (Fig. 4.8) did not show typical spectra of lignified cell walls with maximum absorbance at 275-280 nm (Iiyama and Wallis, 1990). Instead, three maximum absorptions were found at 250-260 nm, 310 nm and 350 nm, respectively. Furthermore, the absence of UV-autofluorescence and a negative phloroglucinol test suggested that these walls are unlignified (data not shown). Only tracheid cell walls exhibited strong UV-autofluorescence, and were coloured red after phloroglucinol staining (data not shown).

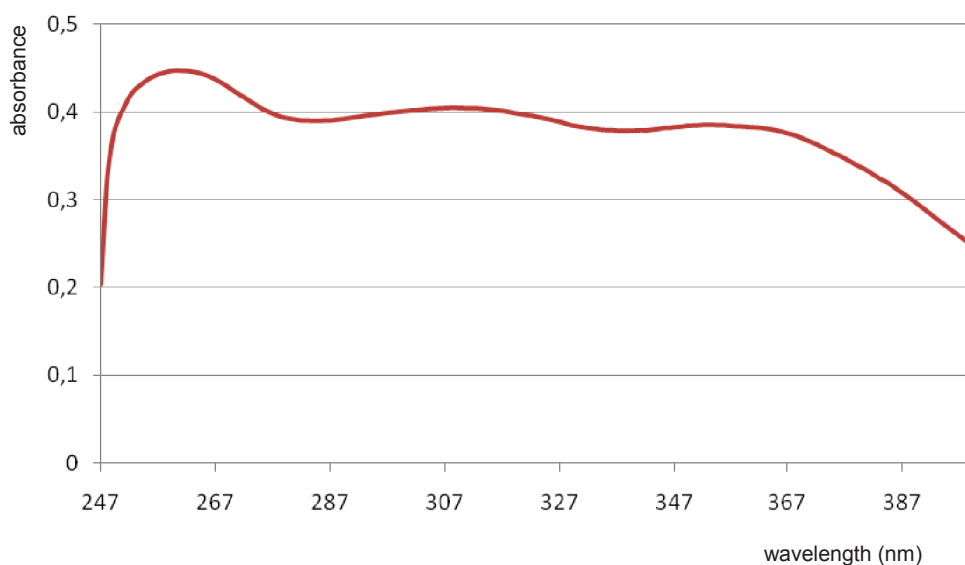


Figure 4.8: UV-spectra of acetyl bromide treated sclereid cell walls of *A. elliotii*. Three maximum absorptions were found at 250-260 nm, 310 nm and 350 nm, respectively. Typical spectra of lignified cell walls (maximum absorbance at 275-280 nm) are absent.

Sugar analysis (Table 4.2) indicated that the totality of neutral and acid monomers represented almost 40% of the cell wall residue. Acid hydrolysis of the residue mainly gave rise to glucose and mannose monomers that represented equal proportion of the total sugars (Table 4.2), but high amounts of rhamnose were also found. Other monomers only accounted for less than 2% of the total sugars.

Table 2: Monosaccharide composition of the sclereid cell walls (presented as % of total monosaccharides released by acid hydrolysis)

Sugars	%
fucose	0,1
arabinose	0,6
rhamnose	21,5
galactose	0,9
glucose	38,4
xylose	1,3
mannose	36,4
galacturonic acid	0,6
glucuronic acid	0,2

Variation and taxonomical implications

Through resin and/or hand-cut sectioning we examined roots of 64 *Asplenium*-species. For each species, we studied several roots, and for observation we only considered roots with fully differentiated metaxylem. Our results are listed in Table 1 and some characters were plotted on a molecular tree (Fig. 4.10) adapted from Leroux *et al.* (2010).

To assess the structural variation of the sclerenchyma sheath, we first compared roots of different relative ages of *A. elliotii* (Viane 7492) and *A. scolopendrium* (Viane 10971). Figure 4.10A shows a transverse section of a young root of *A. elliotii* (Viane 7492) where only inner cortex cells are sclerified. Note that the inner cortex is single layered, but that its oblique transverse cell walls often give the impression of a 'double' layer. When roots mature, additional outer cortex cells sclerify leading to a multilayered sclerenchyma sheath (Fig. 4.11B, C). In some species we observed up to five additional sclerified outer cortex layers (data not shown). Similar results were obtained for *A. scolopendrium*. These observations show that the number of sclereid layers is variable. However, there is a tendency that especially small roots only have one, rarely two, layers of sclereids, whereas thick roots can have up to six layers.

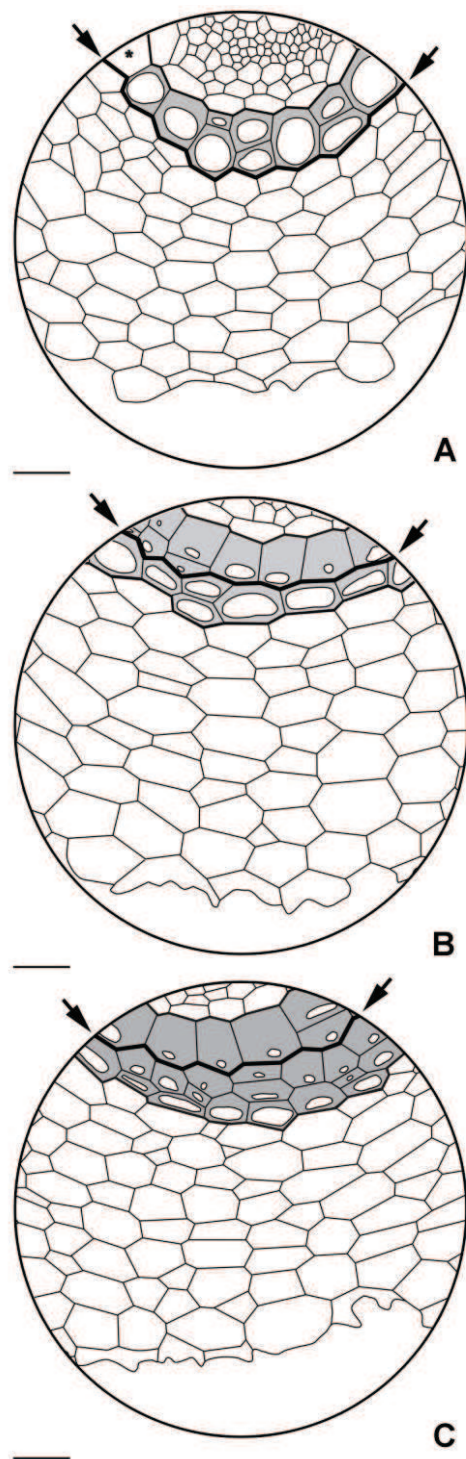


Figure 4.9. Transverse section of an *A. elliotii* root showing different developmental stages of the sclerenchyma sheath. A. Moderate sclerification of inner cortex cells of a section close to the root meristem. Passage zone indicated with an asterisk. **B.** Inner cortex cells are more heavily thickened and adjacent outer cortex cells moderately thickened. The border between sclerified inner cortex and outer cortex cells is indicated with both bold lines and arrows. **C.** Section close to rhizome showing sclerenchyma sheath consisting of heavily thickened inner and outer cortex cells. Scalebars: 50 μ m.

Comparison of sections of several mature roots for each of species studied showed variation in the number of sclereids of the inner cortex. The highest numbers of inner cortex sclereids were found in the thickest roots (Fig. 4.11B, C, F). The number of sclereids was consistent and restricted to only six in species which group together in a monophyletic lineage comprising *A. majus* through *A. crinicaule* (Fig. 4.11D). In these species, sclerified outer cortex cells are always smaller (Fig. 4.11D). It is of interest that in the clade of South-American species comprising *A. juglandifolium* through *A. auritum*, the inner cortex sclereids of most of these species are 1.5 to 3 times larger (Fig. 4.11A) compared to those of adjacent outer cortex sclereids. Similarly, inner cortex sclereids were also radially longer in most species belonging to the clade including *A. theciferum* and *A. obtusatum* (Fig. 4.11C). However, this difference in dimensions was not a consistent pattern within each of these clades, and even within the same root system. A sclerenchyma sheath consisting of sclereids with extremely thickened inner tangential cell walls, is a synapomorphy of the *Asplenium* clade (Fig. 4.10; Fig. 4.11A, B, C, D, E). All *Hymenasplenium* species, on the other hand, are characterized by uniformly thickened sclereids (Fig. 4.11F). Passage zones were found in all the species investigated.

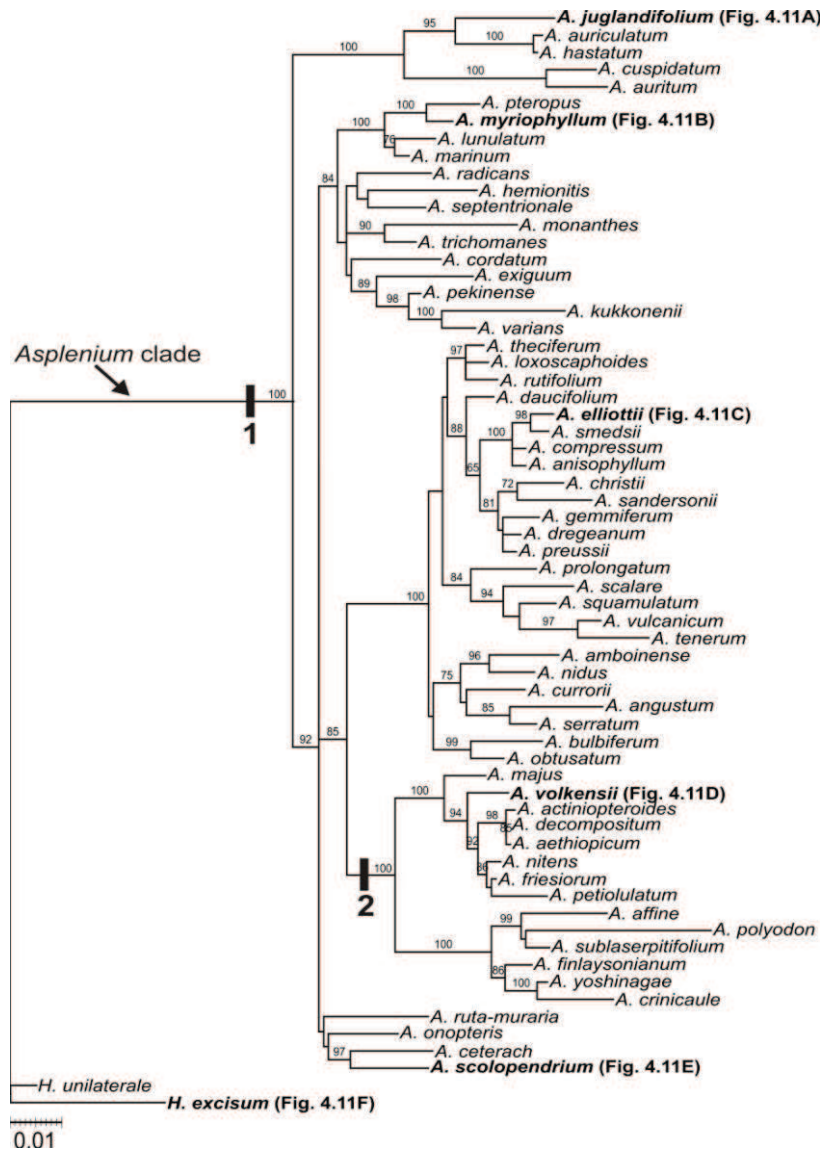


Figure 4.10: Maximum likelihood phylogeny adapted from Leroux *et al.* (2011) based on 64 rbcL sequences. Bootstrap values higher than 70% noted above branches and corresponding drawings of figure 4.11 indicated. Two synapomorphies of the sclerenchyma sheath structure are indicated: 1, unevenly thickened root sclereids; 2, inner cortex consisting of only six cells.

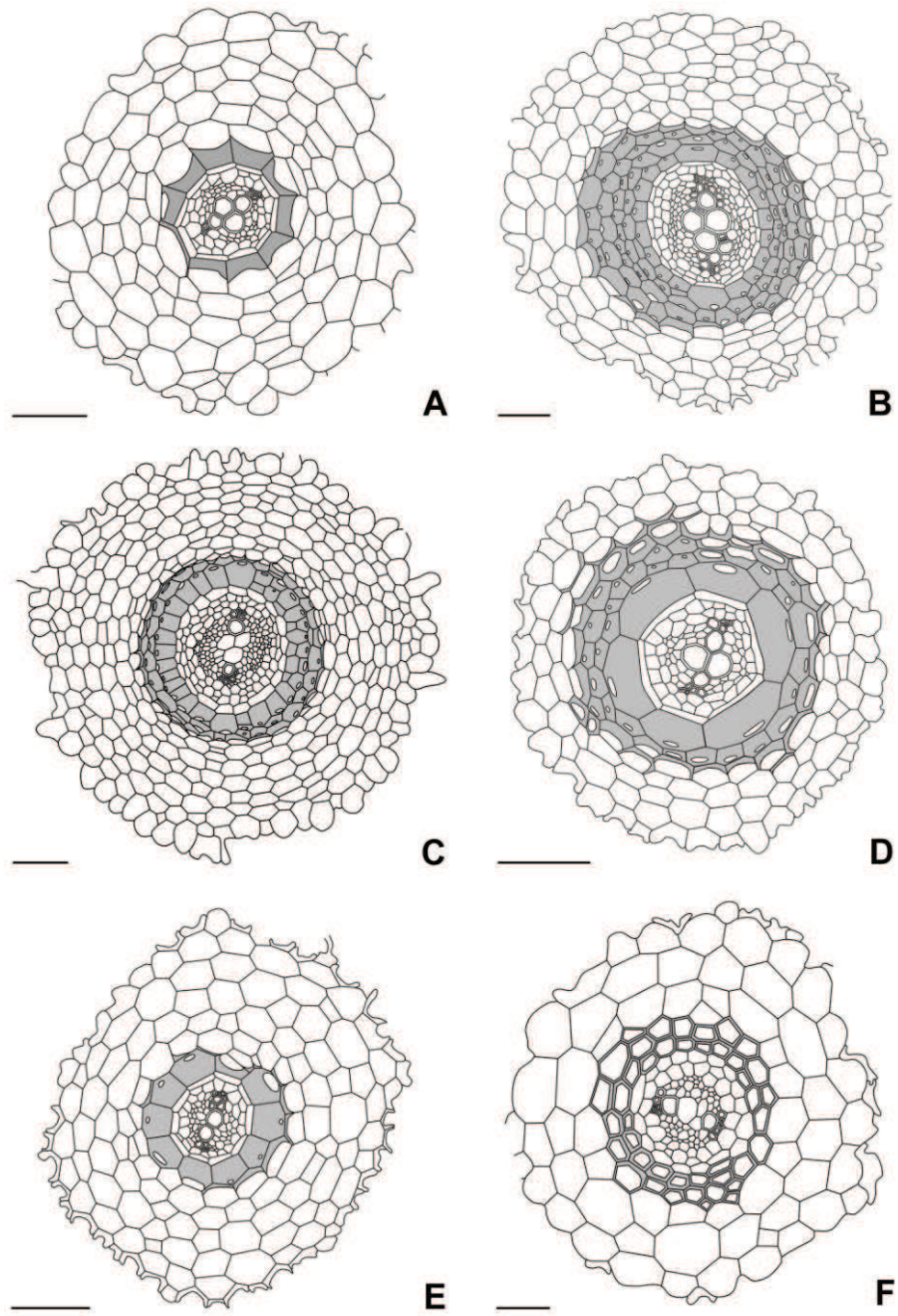


Figure 4.11: Drawings of transverse root sections. A. *A. juglandifolium* (Viane 10667), B. *A. myriophyllum* (Viane 10147), C. *A. elliotii* (Viane 7492), D. *A. volkensis* (Viane 7313), E. *A. scolopendrium* (Viane 10791) and F. *H. excisum* (Viane 11263). Scalebars: 100 μm .

4.4 DISCUSSION

Histogeny, structure and cell wall composition

There is a remarkable level of anatomical precision during the early development of merophytes in leptosporangiate ferns (e.g., Gunning *et al.*, 1978; Barlow *et al.*, 1982; Gifford, 1991). Each daughter cell (merophyte) of the apical cell proceeds through a regular series of formative cell divisions. The early cell divisions in proximal merophytes in *A. bulbiferum* were discussed by Gifford (1991), but in our study, more attention was given to the histogeny of the sclerenchymatous sheath.

Origin of the sclerenchyma sheath

The cortex, including parenchymatous and sclerenchymatous tissues, arises from derivatives of both the inner and the outer cells of the proximal merophytes, as it is the case in most fern species (e.g., Gifford, 1991; Hou & Hill, 2004). Schneider (1997) mentioned that in some *Asplenium* species 'the inner cortex cells divide in anticlinal and periclinal directions, forming a multilayered inner cortex'. Likewise, Gifford (1992) stated that 'the inner cortex of *A. bulbiferum* may undergo periclinal divisions'. In contrast, our study revealed that the inner cortex remains single layered, and that any multilayered sheath includes one to several layers of sclereids derived from the outer cortex. For that reason, we reject the hypotheses of both Schneider (1997) and Gifford (1991). The occasional multilayering observed in inner cortex cells is caused by transverse sections through strongly oblique transverse cell walls between superimposed inner cortex cells, and could have been mistakenly interpreted as being the result of periclinal divisions. Notwithstanding the fact that the inner cortex in Aspleniaceae does not undergo periclinal divisions, in other leptosporangiate ferns such as *Marsilea* (Lin and Raghavan, 1991) a multilayered inner cortex has been observed, and Hou and Hill (2004) stated that, in *Ceratopteris richardii*, the inner and outer cortex undergo periclinal divisions. As our ontogenetical studies were limited to a few species only we cannot exclude that, especially in thick roots, inner cortex precursors might undergo periclinal divisions. Nevertheless, in our study we included species with some of the thickest roots within the Aspleniaceae, such as *A. elliottii*, *A. theciferum*, and *A. compressum*, and we never observed any periclinal divisions in the inner cortex.

Number of sclereids in the inner cortex

We showed that the number of sclereids in the inner cortex depends on the number of additional radial divisions in the inner cortex precursor cells. In *Asplenium aethiopicum* and related species no additional radial divisions occur and six large inner cortex cells remain. In all other species, additional sclereids are formed by random radial divisions in the inner cortex precursor cells. Interestingly, we showed that the single inner cortex cells of the sextants facing the protoxylem poles never undergo radial divisions and this phenomenon is most probably related to the development of passage zones.

Distribution of passage zones

We defined the interruptions of the sclerenchyma sheath as ‘passage zones’ since (1) ‘passage cells’ are used to describe unicellular interruptions in the endodermis or exodermis (Esau, 1965), and (2) passage zones are multicellular structures comprised of unthickened parenchymatous cells flanked by sclereids with increasing cell wall thickness. Schneider (1997) only found passage zones in the roots of some distantly related species, claiming that the presence of this character is the result of parallel evolution. However, we reject this hypothesis, as X-ray computed tomography showed rows of successive passage zones opposite both protoxylem poles and subsequent screening within Aspleniaceae revealed their presence in the roots of all species studied. Passage zones were found in other distantly related fern genera such as ‘*Acrostichum*’, ‘*Aspidium*’, *Pyrrosia* (Ogura, 1972) and *Platynerium* (Ogura, 1972; Chapple and Peterson, 1987). Schneider (1996) reported passage zones in species belonging to the Aspleniaceae, Davalliaceae, Elaphoglossaceae, Lomariopsidaceae, Dryopteridaceae, Hymenophyllaceae, Polypodiaceae, Pteridaceae and Grammitidaceae. The universal occurrence of passage zones in distantly related genera suggest that they play an important structural role and, for example, could provide gaps which offer less resistance to lateral root emergence. However, lateral roots do not develop at each passage zone, indicating that these may alternatively serve as a symplastic pathway of water transport between the cortex and stele.

Origin of the endodermis and pericycle

Whereas most leptosporangiate ferns have a precursor cell common to the endodermis and inner cortex (Gunning *et al.*, 1978; Gifford, 1991; Hou & Hill, 2004), *Equisetum* roots are atypical as their endodermis and pericycle have a common origin (Nägeli and Leitgeb, 1868; Gifford, 1993). Moreover, lateral root initiation in *Equisetum* occurs in the ‘pericycle’, while in other ferns, including the species studied here, the lateral roots arise

from the 'endodermis' (Esau, 1965; Guttenberg, 1966; Lin & Raghavan, 1991; Hou & Hill, 2002; Hou & Hill, 2004). The border between the cortex and stele is situated between the endodermis and the pericycle, as the endodermis is derived from the same lineage as the cortex and the pericycle belongs to the central stele (van Tieghem, 1888a). van Tieghem (1888b) introduced the term 'double endodermis' to avoid the usage of 'pericycle' in *Equisetum*. Topologically, any layer adjoining the vascular tissue should be called pericycle, and in the case of *Equisetum* roots, lateral roots hence develop from the pericycle. However, as the pericycle and the endodermis in *Equisetum* have a common origin, we could adopt the interpretation of van Tieghem (1888b) and accept the existence of a double endodermis, and in that case, lateral roots develop from the 'endodermis'.

Ultrastructure and composition of the sclereid cell wall

Electron and fluorescence microscopy showed that the sclereid cell wall exhibits a polylamellate structure consisting of lamellae with alternating microfibril orientation and composition. The different levels of autofluorescence intensity and the differences in electron-density also suggested that the lamellae may vary in composition. We demonstrated that the use of ADF-STEM has great potential to investigate cell wall morphology at higher resolutions as standard electron microscopy stains - which are necessary for conventional BF-TEM - such as osmium tetroxide and uranyl acetate, primarily stain proteins, membranes, and nucleic acids, and to a far lesser degree carbohydrates. This indicates that only a very low contrast of cell wall polysaccharides can be obtained by BF-TEM (Sarkar *et al.*, 2008), unless (1) polysaccharides can be stained selectively with a high electron contrast reagent, or (2) other techniques such as ADF-STEM are employed.

The solid-state CP-MAS ^{13}C NMR results suggest that lignins are not present in any consistent amount in the sclereids as their characteristic NMR pattern did not appear in the ^{13}C spectrum. If lignin is present, its proportion is extremely low compared to the predominant flavonoid tannin content in such way that its NMR pattern could be masked. In addition, acetylbromide analysis of sclereids did not provide evidence for the presence of lignins and further suggested that sclereids in Aspleniaceae have non-lignified secondary cell walls. Lignins have been reported to occur in ferns (Sarkanen and Hergert, 1971; Chen, 1991), but there is no published information on the lignin-content of wall preparations solely obtained from sclerenchymatous cell walls, and therefore the detected lignins were assumed to occur in all secondary cell walls, including those of the sclerenchyma. The absence of UV-autofluorescence of sclereid cell walls also suggested that these walls are non-lignified. However the presence of phenolic compounds could

have blocked UV-autofluorescence. The strong UV-autofluorescence, in conjunction with a positive phloroglucinol staining, suggested that only tracheids are lignified.

The presence of considerable amounts of carbohydrates is confirmed by the dominating NMR bands in the 60 ppm to 80 ppm range. The great majority, possibly the totality, of these carbohydrates appeared to be hemicellulosic. The origin of these carbohydrates could either be due to the presence of hydrolysable tannins, polymeric materials with a repeating pentagalloylglucose unit, or to the presence of glucomannans that are a very important class of hemicelluloses frequently mixed with flavonoid tannins. However, acid hydrolysis of the residue mainly gave rise to glucose and mannose monomers that represent equal proportion of the total sugars, thereby indicating the predominance of glucomannans. Moreover, immunocytochemistry confirmed the presence of mannans, as the LM21 antibody bound to sclereid cell walls. To some extent the sugar composition is similar to that of gymnosperm secondary cell walls where glucomannans are also the major hemicellulosic compounds (Shimizu, 1991; Whistler and Chen, 1991). In contrast, the major non-cellulosic polysaccharide in secondary cell walls of dicots are xylans (Harris, 2005), and in our study, we did not obtain strong evidence for the presence of high amounts of xylans. Indeed, xylose monomers, reflecting the occurrence of xylans, represented only a very weak proportion, and we did not obtain binding to sclereid cell walls with the LM11 xylan antibody. High quantities of mannans have been detected in fern cell walls, including the eusporangiate genera *Psilotum* and *Equisetum* and the leptosporangiate genus *Pteridium* (Timell, 1964; Bremner and Wilkie, 1971; Popper and Fry, 2004), suggesting an important structural role for glucomannans in strengthening (secondary) cell walls of early-diverging tracheophytes.

We found evidence for the presence of highly polymerized polyflavonoid tannins. Potential acid-induced transformation of these tannins could lead to reddish-brown coloured phlobaphenes (Malan and Chen, 1992), which have been proposed to be responsible for the observed dark colours of some fern stipes by Ogura (1972). Acid hydrolysis of the solvent-extracted residue led to a high amount of rhamnose that accounted for more than 20% of the total sugars. These rhamnose monomers can be attributed to the glycoside moieties of the polyphenolic compounds or to the presence of pectic polysaccharides. However, arabinose, galactose and galacturonic acids, which are typical monomers of pectins (Willats *et al.*, 2006), represented less than 1% of the total sugars, and this suggests the absence of pectic polysaccharides in the sclereid cell walls. Moreover, none of the pectin antibodies (JIM5, JIM7, LM5 and LM6) bound to the sclereid

cell walls. The characteristic colour of the sclereids is mainly caused by the quinine functions derived by oxidation of the phenolic hydroxygroups of the flavonoid tannins.

Variation and taxonomical implications

By studying roots of different relative ages from the same plant, we found that the number of sclereid layers in the root sclerenchyma sheath has no taxonomical significance. As reported by Schneider (1997) the mode of cell wall thickening of the sclereids supports the delimitation of *Asplenium* and *Hymenasplenium*. All *Hymenasplenium*-species were shown to have uniformly thickened sclereids, whereas cell walls of *Asplenium* sclereids are unevenly thickened. Rumpf (1904) first reported that some *Asplenium*-species only have six inner cortex cells and Schneider (2004) was the first to indicate that all these species form a monophyletic lineage. In all other clades, the number of sclereids in the inner cortex was variable at species-level. Schneider (1997) only found passage zones in the roots of some distantly related species, claiming that the presence of this character is the result of parallel evolution. However, we reject this hypothesis, as we found passage zones in all species studied.

4.5 CONCLUSIONS AND FUTURE PERSPECTIVES

In order to use anatomical characters as a source of systematically useful data, it is necessary to understand the range and source of character variation within an individual plant, species or group of related taxa. In this paper we focused on the histogeny and structure and taxonomical significance of the sclerenchyma sheath in Aspleniaceae roots. Our results indicated that the sclerenchyma sheath in Aspleniaceae originates from two early-separated ontogenetic cell-lineages and that the number of sclereid layers has no taxonomical significance. Moreover we showed that the inner cortex is always single layered and the occasional observation of periclinal divisions in the inner cortex is to be explained by their oblique transverse cell walls. We found passage zones in all Asplenioid species and, consequently, we rejected the hypothesis of Schneider (1997) that they are the result of parallel evolution. They represent gaps (1) which offer less resistance to lateral root emergence, and/or (2) serve to transport water from the cortex to the stele. Despite the fact that sclerenchyma sheath in shows some taxonomical insignificant structural variation, we confirmed some of the results of Schneider (1997, 2004). Firstly, all species with six inner cortex cells are grouped together in one monophyletic clade, and secondly, the mode of cell wall thickening allows discrimination of *Asplenium* from *Hymenasplenium*.

Our detailed study of the (ultra)structure and composition of the sclerenchyma sheath of *A. elliotii* provided evidence for the presence of non-lignified secondary cell walls in ferns as we failed to detect lignins by solid-state CP-MAS ^{13}C NMR and acetylbromide analysis. Our NMR analysis further suggested that hemicellulosic glucomannans dominated by far and this was supported by (1) the high amount of mannose monomers found after acid hydrolysis of cell wall residue, and (2) the binding of the anti-mannan LM21 antibody to the sclereid cell walls. The characteristic yellowish-brown colour is most probably caused by oxidation of polyflavonoid tannins. Annular dark field scanning transmission electron microscopy (ADF-STEM) showed the polylamellated nature of the sclereid cell walls and both the (1) variability in electron-density and (2) different intensity levels of red autofluorescence suggest that the lamellae may vary in molecular composition.

Comparative studies are needed at higher taxonomical levels, preferably within ferns and/or lycophytes and should focus on the origin of the root cortex, the functional role of passage zones, and the distribution of non-lignified secondary cell walls. It is of interest to note that, as in angiosperm roots, positional information often predominates over cell lineage information and merophyte boundaries in tissue pattern formation in fern roots (Hou and Blancaflor, 2009). For instance, most fern roots have a diarch stele originating from a three-fold rotational symmetrical root body produced by derivatives of the three proximal sides of the apical cell. In addition, cells of the pericycle lineage can differentiate into sieve elements as reported for *Azolla* and *Ceratopteris* (Gunning *et al.*, 1978; Hou and Hill, 2004) and in this study we showed that sclerification is not restricted to the inner cortex, but also occurs in adjacent outer cortex cells. Detailed genetic studies will provide insights into the nature of the positional signals and how they regulate differentiation of tissues and cell types in derivatives of the single apical cell (Hou and Blancaflor, 2009). It has also been suggested that the developmental program for lateral root initiation in fern roots might be different compared to angiosperm roots (Hou *et al.*, 2004). In light of the discussed differences regarding lateral root initiation between *Equisetum* and most leptosporangiate ferns, future (comparative) studies should also focus on *Equisetum* and the homology of the 'endodermis' in land plants. Moreover, detailed developmental studies are still lacking, especially for lycophytes, which show a greater diversity of root apical organisation compared to ferns (von Guttenberg, 1966). Molecular developmental genetics will be very helpful for revealing tissue and organ identity and origin, and should be extended to studies on ferns and lycophytes.

4.6 ACKNOWLEDGEMENTS

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5

Non-lignified helical cell wall thickenings in root cortical cells of Aspleniaceae (Polypodiales): histology and taxonomical significance

This chapter is a modified version of:

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Summary

Extraxylary helical cell wall thickenings in vascular plants are not well documented except for those in orchid velamen tissues which have been studied extensively. Reports on their occurrence in ferns exist, but detailed information is missing. The aim of this study is to focus on the broad patterns of structure and composition and to study the taxonomic occurrence of helical cell wall thickenings in the fern family Aspleniaceae. Structural and compositional aspects of roots have been examined by means of light, electron, epifluorescence and laser scanning confocal microscopy. To assess the taxonomical distribution of helical cell wall thickenings we performed a molecular phylogenetic analysis based on *rbcL* sequences of 64 taxa. The helical cell wall thickenings of all examined species showed considerable uniformity of design. The pattern consists of helical, regularly bifurcating and anastomosing strands. Compositionally, the cell wall thickenings were found to be rich in homogalacturonan, cellulose, mannan, and xyloglucan. Thioacidolysis confirmed our negative phloroglucinol staining tests, demonstrating the absence of lignins in the root cortex. All taxa with helical cell wall thickenings formed a monophyletic group supported by a 100% bootstrap value and composed of mainly epiphytic species. In conclusion, this is the first report of non-lignified pectin-rich secondary cell walls in ferns. Based on our molecular analysis we reject the hypothesis of parallel evolution of HCWTs in Aspleniaceae. HCWTs can mechanically stabilize the cortex tissue, allowing maximal uptake of water and nutrients during rain fall events. In addition, it can also act as a boundary layer increasing the diffusive pathway towards the atmosphere, preventing desiccation of the stele of epiphytic growing species.

5.1 INTRODUCTION

The cosmopolitan family Aspleniaceae is one of the most species-rich among leptosporangiate ferns with over 720 terrestrial, epilithic or epiphytic ferns (Kramer & Viane, 1990). Most researchers today recognize *Asplenium* and *Hymenasplenium* as well supported segregate genera and some of them have shown that satellite genera such as *Camptosorus*, *Loxoscaphe*, *Diellia*, *Pleurosorus*, *Phyllitis*, *Ceterach* and *Thamnopteris* are nested within *Asplenium* s.l. (Murakami *et al.*, 1999; Gastony & Johnson, 2001; Van den Heede *et al.*, 2003; Schneider *et al.*, 2004; Bellefroid *et al.*, 2010).

During a comparative anatomical survey of Aspleniaceae we observed helical cell wall thickenings (HCWTs) in the root cortex cells of a number of *Asplenium* species. Cortex cells with helical thickenings have been reported to occur in epiphytic ferns (Ogura, 1972). In Aspleniaceae, HCWTs were previously studied by Schneider (1996, 1997). He found that some epiphytic *Asplenium* species such as *A. theciferum* possessed HCWTs while other epiphytes such as *A. nidus* lacked this cell wall feature. Based on the observation that HCWTs seemed to occur in other non-related species, he suggested that HCWTs are possibly the result of parallel evolution.

In extraxylary tissues, HCWTs are less known with the exception of the well documented multiseriate rhizodermis in many orchid roots first described by Link (1824) and designated as the *velamen radicum* by Schleiden (1849). The specialised velamen tissue arises from the protoderm and at maturity it consists of multiple layers of dead cells (Leitgeb, 1864). Since Schleiden (1849), most consider the velamen to be an indirect or direct water-absorbing structure (Duchartre, 1856; Haberlandt, 1909; Eames & McDaniels, 1925; Barthlott & Capesius, 1975; Haas, 1975; Benzing *et al.*, 1983; for a review see Pridgeon, 1987), however, others believe that its primary function is preventing water loss as well as providing mechanical support (Dycus & Knudson, 1957). In a scanning electron microscope survey of the velamen anatomy of Orchidaceae, Porembski & Barthlott (1988) drew attention to the occurrence of tracheoidal cells, forming velamen-like tissues in the root cortex. They called this partially or totally modified root cortical tissue “pseudovelamen”. The arrangement of the thickenings and the post mortal stability of the tissue suggested that it has a mechanical function (Burr and Barthlott, 1991). Other, less known, examples of HCWTs in non-vascular tissues are tracheoidal idioblasts in leaves of certain orchids and in a number of dicotyledonous genera (Pirwitz, 1931; Foster, 1956; Ter Welle, 1975; Saadeddin & Doddema, 1986; Olatunji & Nengim, 1980) as well as in elaters of some liverworts and hornworts (Kremer & Drinnan, 2003).

In ferns, HCWTs were also briefly reported in the root cortex cells of *Pyrrosia* (Polypodiaceae) (Giesenhagen, 1901, Pande, 1935), Pteridaceae (Schneider, 1996), Vittarioid genera (Pteridaceae) and grammitid genera (Polypodiaceae) (Schneider, 1996).

In this paper we present a comparative study on HCWTs in the root cortex of Aspleniaceae using light and scanning microscopy. A more detailed histological study, using epifluorescence and transmission electron microscopy, was conducted on root cortex tissues of *A. elliotii* and *A. daucifolium*. We have also examined the taxonomical relevance of these structures in Aspleniaceae by plotting this character on a molecular tree based on *rbcL* sequences. Possible functions for these cell wall modifications in root cortical cells are discussed.

5.2 MATERIAL AND METHODS

5.2.1 Material

Fresh roots were obtained from our living collection kept at the Ghent Botanical Garden, Belgium. All species used for the histological and molecular studies are listed in Table 5.1 and Appendix 1 respectively.

5.2.2 Light Microscopy

Phloroglucinol/HCl staining was performed by staining fresh hand-cut sections with 2% phloroglucinol in 95% ethanol for 5 min and subsequent mounting in 33% hydrochloric acid. Preparations were viewed with a Zeiss Axioplan II microscope equipped with phase contrast optics. For detailed observation of HCWTs, roots were fixed in FAA (formalin 5% v/v, 50% ethanol v/v, 5% acetic acid v/v), dehydrated in a graded ethanol series, embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany), sectioned, stained and mounted following Leroux *et al.* (2007a). Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

5.2.3 Immunohistochemistry and fluorescence staining

For transverse sections, roots were clamped in between two sheaths of Parafilm M (Pechiney Plastic Packaging Inc, Neenah, WI, USA) and sectioned using a single edged razor blade.

To study the HCWTs on cross walls of the outer cortex we made permanent slides for repeated observation with laser scanning confocal microscopy. Sections were

bleached with 10% sodium hypochlorite, briefly rinsed in 45% acetic acid and stained overnight under vacuum in a safranin staining solution (1% w/v safranin O, 2% w/v formalin and 1% w/v sodium acetate in 1:1 methylcellulose:50 % ethanol). Sections were washed at least three times in acidified 50% ethanol until no further colour was washed from the sections. After dehydration in a graded ethanol series and a subsequent xylene intermedium, sections were mounted in Histomount (National Diagnostics, Atlanta, GA, USA). Slides were viewed using a laser scanning confocal microscope (Nikon e-C1 mounted on a Nikon TE2000). After exciting the staining signal at 543 nm using a HeNe laser and detecting the fluorescence emission with a 571 nm long pass filter, photos were taken at an X/Y resolution of 512x512 pixels. Z-stacks were made every 0.4 μm and combined in one X/Y image.

For indirect immunofluorescence labelling, hand-cut sections were obtained as described above. A range of probes directed to cell wall polysaccharides was used to check the presence of some major cell wall polymers in HCWTs. These included the anti-homogalacturonan monoclonal antibodies JIM5, JIM7 and LM19 (Clausen *et al.*, 2003; Verhertbruggen *et al.* 2009); anti-1,4-galactan antibody LM5 (Jones *et al.*, 1997), anti-1,5-arabinan antibody LM6 (Willats *et al.*, 1998); anti- α -D-glucanase antibody LM1 (Smallwood *et al.*, 1995), anti-xylan antibody LM11 (McCartney *et al.*, 2005), anti-xyloglucan antibody LM15 (Marcus *et al.*, 2008), anti-mannan monoclonal antibody LM21 (S.E. Marcus and J.P. Knox, unpubl. res.) and a his-tagged carbohydrate-binding module CBM3a directed to crystalline cellulose (Blake *et al.*, 2006).

Immunolabeling was performed as described elsewhere (Leroux *et al.*, 2007b and Blake *et al.*, 2006). Immunofluorescence was observed with a microscope equipped with epifluorescence irradiation (Olympus BX-61) and images were captured with a Hamamatsu ORCA285 camera and prepared with Improvision Volocity software. Thicker hand-cut sections were observed using a laser scanning confocal microscope (Nikon e-C1 mounted on a Nikon TE2000). After exciting the staining signal at 488 nm using an Argon laser and detecting the fluorescence emission with a 540 nm long pass filter, photos were made at an X/Y resolution of 512x512 pixels. Z-stacks were made every 0.4 μm and combined in one X/Y image.

5.2.4 Scanning electron microscopy

Longitudinal hand-cut sections of roots were fixed in FAA, dehydrated in a graded ethanol series followed by a graded acetone series, and dried in a critical point dryer (Balzers CPD-030) using CO_2 as a transition fluid. Dried sections were mounted on clean aluminium stubs with double-sided adhesive graphite tabs. Mounted specimens were

coated with gold (12-15 nm thick) using Balzers SPD-050 sputter coater. Sections were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

5.2.5 Transmission electron microscopy

Root cortex tissue was dissected in order to produce blocks measuring approximately 5 mm length at all sides. They were fixed with 2% w/v paraformaldehyde and 2% v/v glutaraldehyde in a cacodylate buffer 0.1 M pH 6.9 for 24 h at 4°C, post fixed in 2% osmium tetroxide for 3 h, washed in the same buffer and dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100% alcohol/Spurr's resin (1:1) at 4°C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4°C), and transferred to 100% Spurr's resin and left overnight at 4°C. Polymerization was performed at 70°C for 16 h. Seventy-nm-thick sections were made using a Reichert ultracut S Ultramicrotome (Leica, Vienna, Austria). Formvar-coated single slot copper grids were used. Sections were stained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 10 min in lead citrate stain at 20°C. The grids were examined with a JEM-1010 electron microscope (Jeol Ltd, Tokyo, Japan) equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

5.2.6 Lignin analysis

Both whole root and isolated outer cortex tissue were milled to 0.5 mm prior to removal of extractives by successive treatment by 80% ethanol at 40°C and acetone (room temperature). Samples were dried and submitted to thioacidolysis in order to provide signature of lignin monomers involved in alky-aryl linkages (Lapierre *et al.*, 1993). Ten mg of sample were submitted to a mixture of BF₃/ethanethiol/dioxane for 4 hours at 100°C as detailed previously (Day *et al.*, 2005). The lignin-derived main thioacidolysis monomers were analyzed as their trimethylsilylated derivatives using gas chromatography, with a fused silica capillary DB1 column (30 m x 0.25 mm, 0.25 µm film thickness, SUPELCO) using helium as a carrier gas (1 mL/min). The temperature gradient was 160-200°C at 3°C min⁻¹. The quantitative evaluation of the guaiacyl (G), and syringyl (S) monomers was carried out using tetracosane as internal standard. Detection was carried out with a flame ionization detector.

5.2.7 Phylogenetic analysis

The present molecular phylogenetic analysis comprises 64 taxa of Aspleniaceae, representing all major clades recognized by Schneider *et al.* (2004) (Appendix 1). Four

taxa are included for which Schneider (1997) reported HCWTs: *A. amboiense*, *A. angustum*, *A. obtusatum*, and *A. scalare*. DNA was extracted either using the DNEasy® Plant Mini Kit (QIAGEN), following the manufacturer's instructions or using a specific fern CTAB DNA extraction protocol designed for both silica dried and herbarium quality material (Dempster *et al.*, 1999). A partial region of the *rbcl* gene was amplified with primers aF and cR (Hasebe *et al.*, 1994). The PCR master mix was prepared according to the instructions of the i-Taq(TM) DNA Polymerase kit (iNtRON Biotechnology, Inc., South Korea). Amplifications were carried out on a PTC-100(TM) thermocycler (MJ Research, Inc., U.S.A.) with the following protocol: initial denaturation of 2 minutes at 94°C followed by 35 cycles of 20 seconds denaturation at 94°C, 20 seconds annealing at 57°C, 1 min 45 seconds extension at 72°C and a final extension of 5 min at 72°C. PCR products were purified using the MEGAquick-spin(TM) kit (iNtRON Biotechnology, Inc., South Korea). Both forward and reverse sequence were sequenced by Macrogen, Inc. (South Korea), and assembled and manually edited in Sequence Manipulation Suite (Stothard, 2000). Sequence alignment was executed with ClustalX (Thompson *et al.*, 1997). Maximum Likelihood (ML) analysis was carried out in RAxML v7.0.3 (Stamatakis, 2006b), using the ML Rapid Bootstrapping algorithm for 1000 replicates, implementing the GTRMIX model with parameters optimized for each partition (Stamatakis 2006a,b; Stamatakis *et al.*, 2008). This was followed by a ML tree search to obtain the tree with the highest likelihood score. Three partitioning strategies were tested: (1) one partition, lumping the three codon positions; (2) two partitions, lumping first and second codon positions and separating the third codon position; (3) three partitions, optimizing model parameters for each codon position separately. Character states (table 5.1) were plotted on the phylogenetic tree by hand.

Table 5.1: List of specimens, methods, presence/absence (+/-) of HCWTs. I: immunohistochemistry, L: thio-acidolysis, LM: light microscopy of resin sections, P: phloroglucinol/HCl test, S: scanning electron microscopy, T: transmission electron microscopy.

Taxon	Voucher	Locality	Methods	HCWTs
<i>A. actiniopteroides</i>	Viane 7583	Tanzania, Mount Kilimanjaro, 3820 m alt.	P	-
<i>A. aethiopicum</i> ssp. <i>tripinnatum</i>	Viane 7377	Kenya, Mount Aberdare, 2980 m alt.	P	-
<i>A. aethiopicum</i> ssp. <i>aethiopicum</i>	Viane 7412	Kenya, Mount Elgon, 2830 m alt.	P	-
<i>A. aethiopicum</i> ssp. <i>aethiopicum</i>	Viane 11230	Uganda, Kanungu District, 1350 m alt.	P, S, LM	-
<i>A. affine</i>	Viane 8227	Réunion, Forêt de Bébou, 1550 m alt.	P, S	-
<i>A. anisophyllum</i>	Viane 6402b	Rep. South Africa, Limpopo, Graskop, 1450 m alt.	P	+
<i>A. anisophyllum</i>	Viane 8775	Zimbabwe, Manicaland, Bvumba Mts., 1560 m alt.	P, S	+
<i>A. auriculatum</i>	Viane 10602	Puerto Rico, Sierra de Luquillo, 755 m alt.	P	-
<i>A. auritum</i>	Viane 10251	Venezuela, Merida, Sierra Nevada, 3075 m alt.	P, S	-
<i>A. auritum</i>	Viane 10368	Venezuela, Vargas, El Avila Range, 1550 m alt.	P	-
<i>A. auritum</i>	Viane 10628	Puerto Rico, W of Maricao, 335 m alt.	P	-
<i>A. boltonii</i>	Viane 8221	Réunion, Forêt de Bélouve, 1560 m alt.	P	+
<i>A. boltonii</i>	Viane 8460	Réunion, Cirque de Cilaos, 1490 m alt.	P	+
<i>A. bulbiferum</i>	Unknown	Botanical Garden Ghent University, location unknown	P, S	+
<i>A. centrafricanum</i>	Bellefroid 393	Uganda, Road Kabale-Kisoro, 3100 m alt.	P	+
<i>A. centrafricanum</i>	Bellefroid 392	Uganda, Road Kabale-Kisoro, 3100 m alt.	P, S	+
<i>A. ceterach</i>	Van den Heede 250b	Cyprus, SE of Pentadactylos, 530 m alt.	P	-
<i>A. ceterach</i>	Viane 11640	Italy, E Sardinia, N of Baunei, 5 m alt.	P	-

<i>A. cei</i>	Viane 11201	Uganda, Kanungu Distr., Bwindi, 1500 m alt.	P	+
<i>A. christii</i>	Viane 8792	Zimbabwe, Manicaland, Chirinda Forest, 1180 m alt.	P	+
<i>A. compressum</i>	Viane 8851	St. Helena, Diana Peak, 730 m alt.	P	+
<i>A. cordatum</i>	Viane 11567B	Rep. South Africa, W. Cape, Huisrivierpass, 400 m alt.	P, S	-
<i>A. cordatum</i>	Viane 11569	Rep. South Africa, W. Cape, Huisrivierpass, 500 m alt.	P	-
<i>A. crinicaule</i>	Viane 11304	China, Guizhou, Sandu, Yao Ren Shan, 550 m alt.	P	-
<i>A. currorii</i>	GUBG *79-2824	Ivory coast, Tai, 180 m alt.	P	+
<i>A. cuspidatum</i>	Viane 10108	Venezuela, Trujillo, 1865 m alt.	P	-
<i>A. daucifolium</i> ssp. <i>lineatum</i>	Viane 8220	Réunion, Forêt de Bélouve, 1560 m alt.	P, S, I, T, L, LM	+
<i>A. daucifolium</i> ssp. <i>viviparum</i>	Viane 8445	Réunion, Cirque de Cilaos, 1050 m alt.	P, S	+
<i>A. decompositum</i>	Viane 7688	Tanzania, Kilimanjaro, 2700 m alt.	P	-
<i>A. dregeanum</i>	Viane 11199	Uganda, Kanungu Distr., Bwindi, 1500m alt.	P, S	+
<i>A. elliotii</i>	Viane 7685	Tanzania, Kilimanjaro, 2750 m alt.	P	+
<i>A. elliotii</i>	Viane 6403	Rep. South Africa, Limpopo, Graskopkloof, 1450 m alt.	P, S, I, T, LM	+
<i>A. erectum</i>	Viane 11247	Uganda, Kabale Distr., Bwindi, 2300m alt.	P	-
<i>A. exiguum</i>	Viane 11102	Mexico, Chihuahua prov., 2110 m alt.	P	-
<i>A. finlaysonianum</i>	Viane 9708	Myanmar, Kachin State, E of Puta-O, 835 m alt.	P	-
<i>A. friesiorum</i>	Viane 11248	Uganda, Kabale Distr., Bwindi, 2300 m alt.	P, S	-
<i>A. friesiorum</i>	Viane 8752	Zimbabwe, Manicaland, Nyanga, 1840 m alt.	P	-
<i>A. gemmiferum</i>	Viane 11117	Uganda, Kasese Distr., Rwenzori, 1840 m alt.	P	+
<i>A. hastatum</i>	Viane 10182c	Venezuela, Mérida, 2090 m alt.	P	-
<i>A. hemionitis</i>	BGUG s.n.	Botanical Garden University of Ghent, location unknown	P	-

<i>A. javorkeanum</i>	Van den Heede 89	Italy, Toscana, Monte Freddone, 1320 m alt.	P	-
<i>A. juglandifolium</i>	Viane 10667	Puerto Rico, Toro Negro forest, 950 m alt.	P, S	-
<i>A. kukkonenii</i>	Viane 10886	China, Xizang (Tibet), Nyingtri distr., 2150 m alt.	P	-
<i>A. lobatum</i>	Viane 11477	Rep. South Africa, Limpopo, Graskop, 1390 m alt.	P	-
<i>A. loriceum</i>	Viane 11271	China, Hainan, Qi Zhi Shan, 805 m alt.	P	+
<i>A. loxoscaphoides</i>	Viane 7549	Tanzania, Mt. Meru, 1780 m alt.	P, S	+
<i>A. lunulatum</i>	Viane 6558	Rep. South Africa, Amatole Range, 1000 m alt.	P, S	-
<i>A. macrophlebium</i>	Viane 11213	Uganda, Kanungu Distr., Bwindi, 1575 m alt.	P	+
<i>A. majus</i>	Viane 7427	Kenya, Mt. Elgon, 3560 m alt.	P	-
<i>A. marinum</i>	Viane 10568	Portugal, Madeira, Seixal, 10 m alt.	P, S, LM	-
<i>A. monanthes</i>	Viane 11106	Mexico, Chihuahua prov., 2110 m alt.	P	-
<i>A. myriophyllum</i>	Viane 10147	Venezuela, Sierra de la Culata, 3100 m alt.	P	-
<i>A. nidus</i> s.l.	Viane 11268	China, Hainan, Qi Zhi Shan, 725 m alt.	P	+
<i>A. nidus</i> s.l.	Viane 9629	Myanmar, Kachin, E of Putao, 490 m alt.	P, S	+
<i>A. nidus</i> s.l.	Viane 9630	Myanmar, Kachin, E of Putao, 490 m alt.	P	+
<i>A. nitens</i>	Viane 8203	Réunion, Forêt de Bélouve, 1560 m alt.	P, S	-
<i>A. onopteris</i>	Viane 8078	Spain, La Palma, E of Tijarafe, 1200 m alt.	P	-
<i>A. pekinense</i>	Viane 10031	China, Beijing, Xiang Shan, 180 m alt.	P	-
<i>A. petiolulatum</i>	Viane 8423	Réunion, SE of Dos d'Ane, 1150 m alt.	P	-
<i>A. polyodon</i>	Viane 11217	Uganda, Kanungu Distr., Bwindi, 1575 m alt.	P, S	-
<i>A. preussii</i>	Viane 8798	Zimbabwe, Manicaland, Chirinda forest, 1150 m alt.	P	+
<i>A. preussii</i>	Viane 11193	Uganda, Kasese Distr., Rwenzori, 1900 m alt.	P	+

<i>A. prolongatum</i>	Viane 11314	China, Guizhou, Sandu, Yao Ren Shan, 550 m alt.	P	+
<i>A. pteropus</i>	Viane 10645	Puerto Rico, Cordillera Central, 1000 m alt.	P	-
<i>A. radicans</i>	Viane 10639	Puerto Rico, Cordillera Central, 1000 m alt.	P, S	-
<i>A. rukararense</i>	Viane 11255	Uganda, Kanungu Distr., Bwindi, 2400 m alt.	P	+
<i>A. ruta-muraria</i>	Viane 10040	France, Drôme, Pont-en-Royans, 320 m alt.	P	-
<i>A. rutifolium</i>	Bellefroid 245	Zimbabwe, Bvumba Mts, Bunga Forest, 1634 m alt.	P	+
<i>A. rutifolium</i>	Bellefroid 249	Zimbabwe, Chimanimani Mts, 1572 m alt.	P	+
<i>A. rutifolium</i>	Bellefroid 235	Zimbabwe, Nyanga, Pungwe Falls, 1842 m alt.	P, S	+
<i>A. sandersonii</i>	Viane 7719A	Tanzania, Mt. Kilimanjaro, 2250 m alt.	P	+
<i>A. scolopendrium</i>	Viane 10971	Belgium, E. Flanders, Ghent, 6 m alt.	P, S	-
<i>A. septentrionale</i>	Viane 9578	France, Savoie, Mt. Cénis, 2010 m alt.	P	-
<i>A. serratum</i>	Viane 10607	Puerto Rico, Sierra de Luquillo, 390 m alt.	P	+
<i>A. sertularioides</i>	Bellefroid 351	Uganda, Kasese Distr., Mt. Rwenzori, 3000 m alt	P, S	+
<i>A. sertularioides</i>	Bellefroid 352	Uganda, Kasese Distr., Mt. Rwenzori, 3000 m alt.	P	+
<i>A. smedsii</i>	Viane 7194	Ethiopia, Mt. Batu, 2850 m alt.	P	+
<i>A. smedsii</i>	Viane 7195	Ethiopia, Mt. Batu, 2850 m alt.	P, S	+
<i>A. smedsii</i>	Viane 7707	Tanzania, Mt. Kilimanjaro, 2470 m alt.	P	+
<i>A. squamulatum</i>	Njo s.n.	Indonesia, N. Sumatra	P, S	+
<i>A. sublaserpitifolium</i>	Viane 11266	China, Hainan, Qi Zhi Shan, 725 m alt.	P	-
<i>A. tenerum</i>	Viane 9043	Indonesia, Kalimantan, SE Schwaner Mts., 100 m alt.	P, S	+
<i>A. tenuicaule</i>	Viane 10888	China, Xizang (Tibet), Nyingtri distr., 2100 m alt.	P	-
<i>A. theciferum</i>	Bellefroid 238	Zimbabwe, Bvumba Mts, Bunga Forest, 1610 m alt.	P	+

<i>A. theciferum</i>	Bellefroid 247	Zimbabwe, Chimanimani Mts, 1589 m alt.	P	+
<i>A. theciferum</i>	Viane 10336	Venezuela, Aragua, Colonia Tovar, 1860 m alt.	P, S, LM	+
<i>A. trichomanesssp. trichomanes</i>	Viane 6351	Italy, La Spezia prov., Brugnato, 150 m alt.	P	-
<i>A. trichomanes ssp. quadrivalens</i>	Viane 10519	Russian Fed., Altay, Teletsko Oz., cape Kuvan, 450 m alt.	P	-
<i>A. varians ssp. varians</i>	Viane 10733	China, Yunnan, Xue Shan, 2020 m alt.	P	-
<i>A. varians ssp. fimbriatum</i>	Viane 11521	Rep. South Africa, E. Cape, Stutterheim, 995 m alt.	P	-
<i>A. volkensii</i>	Viane 11124	Uganda, Kasese Distr., Rwenzori, 2655 m alt.	P, S	-
<i>A. vulcanicum</i>	Njo 35	Indonesia, N. Sumatra	P, S	+
<i>A. yoshinagae</i>	Viane 9395	China, Yunnan, Yunlingshan, Zhongxing, 1950 m alt.	P	-
<i>H. excisum</i>	Viane 11263	China, Hainan, Qi Zhi Shan, 600 m alt.	P, S	-
<i>H. obscurum</i>	Viane 11286	China, Hainan, Wu Zhi Shan, 770 m alt.	P, S	-
<i>H. unilaterale</i>	Viane 8344	Réunion, Forêt de Bébour, 900 m alt.	P, S	-

5.3 RESULTS

The distribution of HCWTs in 99 species of Aspleniaceae is shown in Table 5.1, where methods to which these species have been subjected are indicated.

Histology

The mature roots of Aspleniaceae have the characteristic internal structure common to most fern roots. The outermost layer of the roots of *Asplenium*- and *Hymenasplenium*-species is a one cell layer thick rhizodermis. The cortex, enclosing the central stele, is divided into three zones: the outer cortex of thin-walled cells, the inner cortex of brown sclereids, and the endodermis forming the innermost layer (Fig. 5.1A,B). The cells of the outer cortex are somewhat elongated compared to the isodiametric rhizodermis cells (Fig. 5.1B). In both genera the central cylinder is a radial diarch vascular bundle composed of central xylem surrounded by phloem and a parenchymatous pericycle (Fig. 5.1A). Occasionally the sclerified inner cortex cells are interrupted opposite the protoxylem poles.

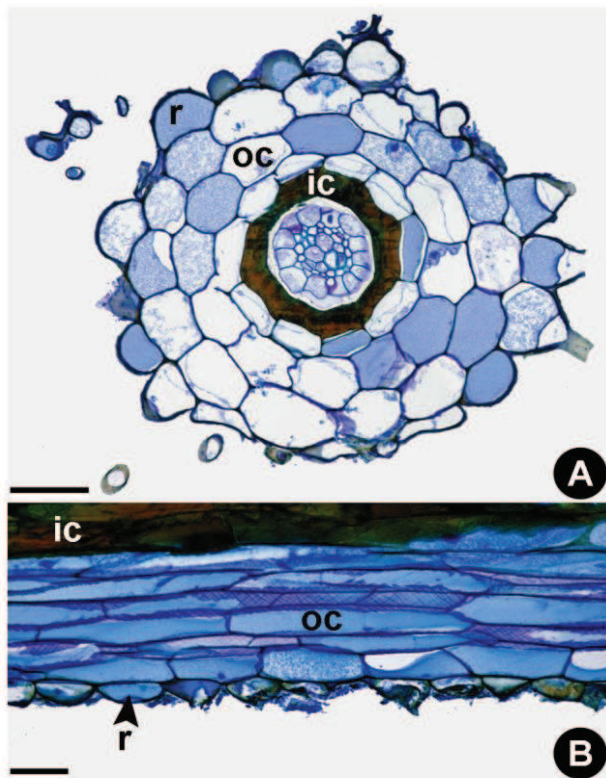


Figure 5.1: Sections of the root of *A. theciferum*. (A) Transverse section showing rhizodermis (r), outer cortex (oc), inner cortex (ic) of sclereids, and central vascular cylinder surrounded by an endodermis. (B) Longitudinal section showing helical cell wall thickenings on several outer cortex cells. Scale bars: 50 µm.

We examined the ontogeny (data not shown) of both the rhizodermis and the cortex in *A. elliotii*, *A. theciferum*, *A. daucifolium*, *A. aethiopicum*, and *A. marinum*. In these taxa the rhizodermis remains a single layer, whereas the outer cortex becomes multilayered. Depending on the number of periclinal divisions in its precursor cells up to nine layers are formed. Unlike the velamen cells of orchids, which die soon after this process, the outer cortex cells of *Asplenium* and *Hymenasplenium* remain alive. The first evidence of the development of HCWTs is found in regions just above the expansion zone, less than 4 mm behind the root apical cell. At about 20 mm from the root apex HCWTs are more prominent, but we did not find a strict correlation between their place of origin and a particular distance above the root tip.

To study the patterns of HCWTs, longitudinal preparations of cortex tissue were examined with a scanning electron microscope (SEM). The HCWTs showed considerable consistency of design. A selection of the results obtained by scanning electron microscopy is presented in figure 5.2. The patterns consist of helical strands which often branch at certain places (Fig. 5.2A,C,E). Occasionally, anastomoses between strands were observed (Fig. 5.2C). No pores or pits were observed in any of the species studied (Fig. 5.2).

Laser scanning confocal microscopy of HCWTs on transverse cross walls in the outer cortex, showed that they have a random pattern: some are arranged in a tangential-parallel pattern, whereas others are distributed more randomly (Fig. 5.4A).

Ultrastructural studies gave similar results for *A. elliotii* and *A. daucifolium*. The profiles of the strands are not as symmetrical as they usually are in xylem elements. Moreover, the strands are unpaired on opposite sides of the common cell wall (Fig. 5.3a). The greater part of the wall deposition is restricted to the helical strands whereas the inter-strand regions undergo no to relatively modest thickening. No intercellular spaces, perforations or pits were observed, which confirms the SEM observations. The primary walls and the middle lamellae are difficult to distinguish and form an electron-dense unresolved zone (Fig. 5.3b). A similar electron-dense zone was found in the cell wall regions between tracheids and adjacent xylem parenchyma cells in the stele (Fig. 5.3d). As xylem parenchyma cells do not have secondary cell walls, the electron-dense zone therefore includes the primary cell walls of the tracheid and xylem parenchyma cells. A similar zone, which will be referred to as compound middle lamella (Esau, 1965), is also visible between tracheids (Fig. 5.3c). The secondary cell walls of the outer cortex cells are somewhat fibrillar in appearance (Fig. 5.3b). A fibrillar layer bulges into the lumen of the cell and is hemispherical, unlike the primary walls which are uniform in thickness. A second layer forms over the wall facing the lumen and seems to be an impregnation of the outer zone of the first fibrillar layer (Fig. 5.3b). The thickness of this impregnated zone

is variable among outer cortex cells and is most likely depending on the level of impregnation.

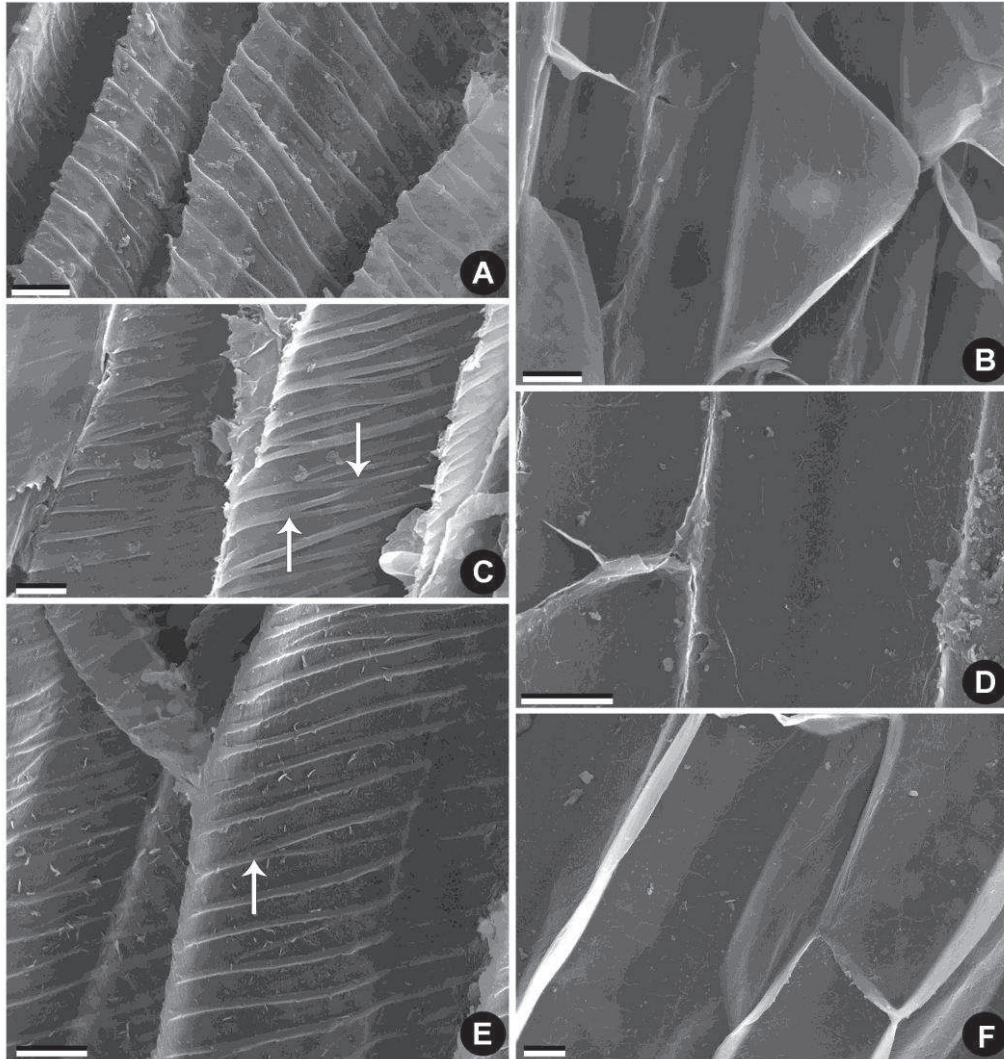


Figure 5.2: Scanning electron micrographs of cell wall surfaces of outer cortex cells of *Asplenium* and *Hymenasplenium* roots, showing presence (A, C, E) or absence (B, D, F) of HCWTs. (A) *A. nidus*; (B) *A. marinum*. (C) *A. elliottii*. (D) *A. lunulatum*; (E) *A. squamosum*.; (F) *H. excisum*. Arrows indicate anastomosing and/or bifurcating strands. Scale bars: 10 μ m.

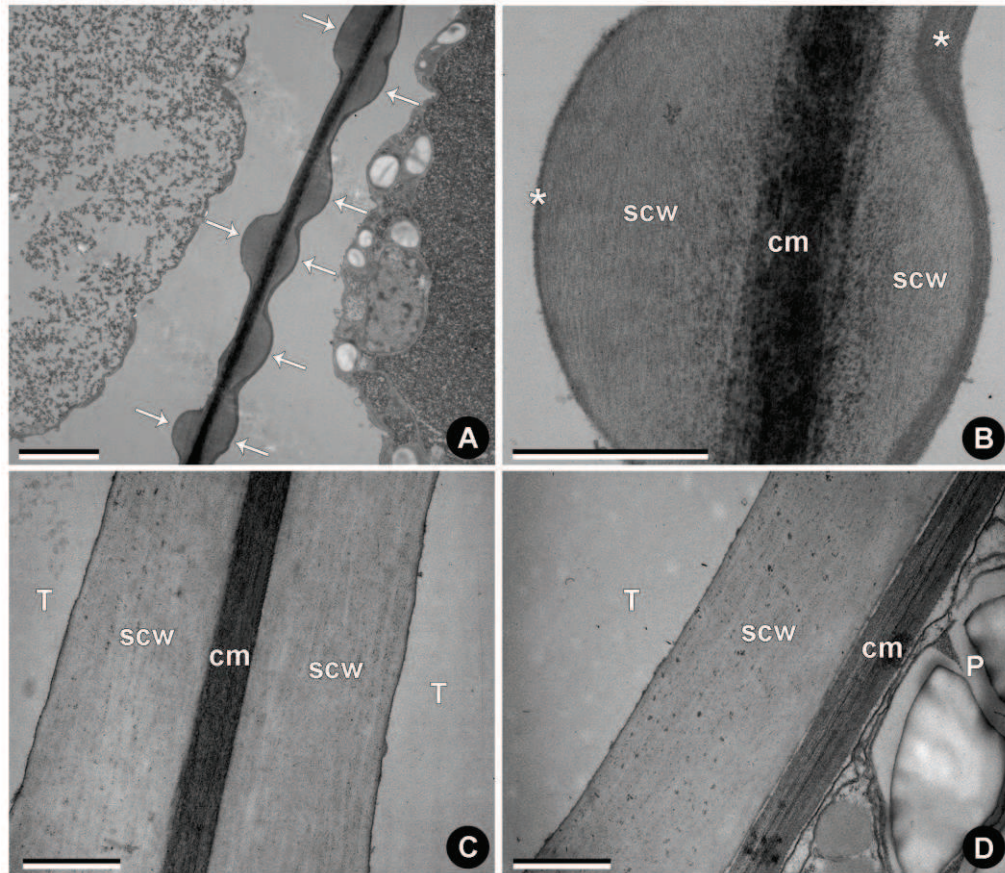


Figure 5.3: Transmission electron images of transverse root sections of *A. elliotii*. (A) Transverse section of outer cortex cell showing HCWTs (arrows) on adjacent cell walls. (B) Detail of (A) showing compound middle lamella (cm), secondary cell walls (scw) with a more electron-dense outer zone (*). (C) Cell walls of adjacent tracheids showing the compound middle lamella (cm) and the secondary cell walls (scw). (D) Cell wall between a tracheid (T) and a xylem parenchyma cell (P). The electron-dense zone (cm) includes the primary cell wall of both cells as well as their intervening middle lamella. Scale bars: A: 3 µm; B: 1 µm; C,D: 500 nm.

Since wall thickenings in orchid velamen-tissue contain lignins, we examined the presence of lignins in *Asplenium* HCWTs. In none of the species studied, HCWTs were stained after phloroglucinol/HCl treatment. Because it was impossible to stain older roots using phloroglucinol due to dark pigments in the cortical cell walls, we traced lignins chemically in *A. daucifolium*, possessing heavily impregnated dark cortical cell walls. Thioacidolysis disrupting the non-condensed inter-monomer linkages (alkyl-aryl ether) enabled the detection of trithioethylated derivatives of guaiacyl (G) and syringyl (S)

monomers at trace levels (90 and 68 $\mu\text{mol/g}$ respectively) in entire root tissues. But in contrast, no such lignin monomers were found in isolated outer cortex tissues.

We used several monoclonal antibodies and a carbohydrate-binding module to examine the presence of some major cell wall constituents in HCWTs of *A. elliotii* and *A. daucifolium* and obtained similar results for both species. The monoclonal antibodies JIM5, JIM7 and LM19, which all bind to a range of partially methyl-esterified homogalacturonan domains, clearly labelled the HCWTs as shown for LM19 in Fig. 5.4B. The CBM3a probe targeting crystalline cellulose (Fig. 5.4C) and the anti-xyloglucan monoclonal antibody LM15 recognising the XGGG-motif (Fig. 5.4D) also bound to these cell wall thickenings. HCWTs also showed a positive reaction after calcofluor staining (Fig. 5.4E). LM21, a monoclonal antibody to mannan, bound weakly to the sclereids of the sclerenchyma sheath and to HCWTs (Fig. 5.4F). The LM5 1,4 $\rightarrow$ galactan, LM1 extensin, LM11 xylan and LM6 1,5 $\rightarrow$ arabinan monoclonal antibodies did not bind to HCWTs. However, LM11 bound to tracheid cell walls, while LM6 labelled all cell walls of the pericycle and vascular parenchyma within the central cylinder. Antibodies such as JIM7 only bound to the cell walls of cortex tissues in very young roots and not in older roots. Very early during maturation, the compound middle lamellae of the root cortex cells become impregnated with yellow-brown pigments. In older roots, with fully developed metaxylem and a thick sclerenchyma sheath surrounding the stele, the HCWTs also become impregnated with such pigments.

***rbcL* based phylogenetic analysis**

The three partitioning strategies resulted in trees with identical topologies and almost equal bootstrap support values. Likelihood scores were lowest for the strategy without partitioning while the other strategies produced trees with better and similar likelihood scores. In each analysis, all species with HCWTs formed a single clade (*A. theciferum* to *A. obtusatum*) with 100% bootstrap support. Figure 5.5 shows the tree resulting from the strategy using two partitions.

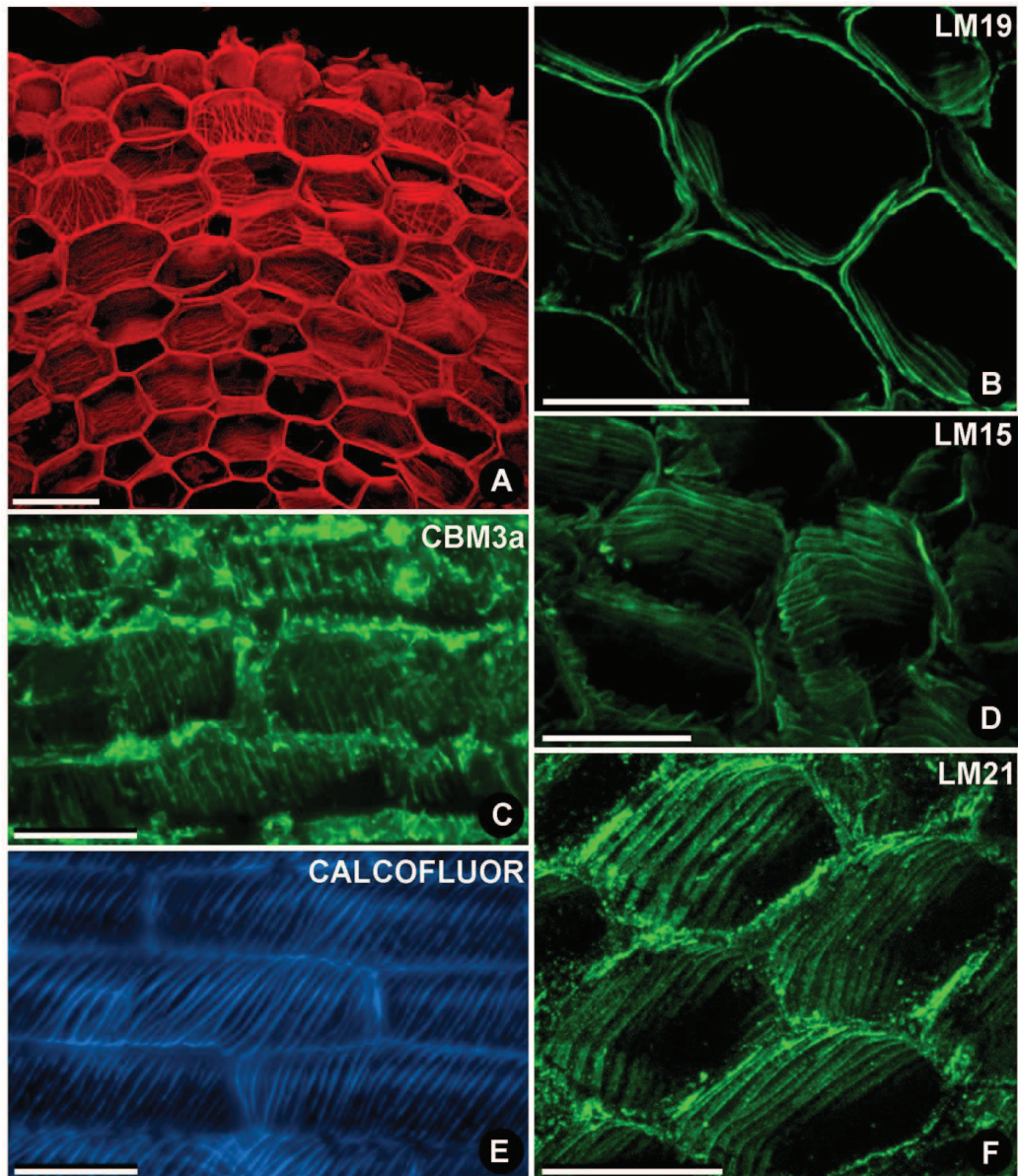


Figure 5.4: Indirect immunofluorescence labelling of cell wall polymers and fluorescent staining of sections of *A. elliotii* roots. (A) Reconstruction of confocal optical sections of safranin stained root showing patterns of cell wall thickenings on transverse walls. (B) Reconstruction of confocal optical sections showing binding of LM19 antibody (anti-homogalacturonan) to HCWTs but not to compound middle lamellae. (C) Longitudinal section showing HCWTs labelled with CBM3a (crystalline cellulose). (D) Reconstruction of confocal optical sections showing LM15 epitope (anti-xyloglucan) on HCWTs. (E) Calcofluor white staining of HCWTs, indicating the abundance of cellulose. (F) Reconstruction of confocal optical sections showing LM21 labelling (anti-mannan) of HCWTs. Scale bars: 50 μm .

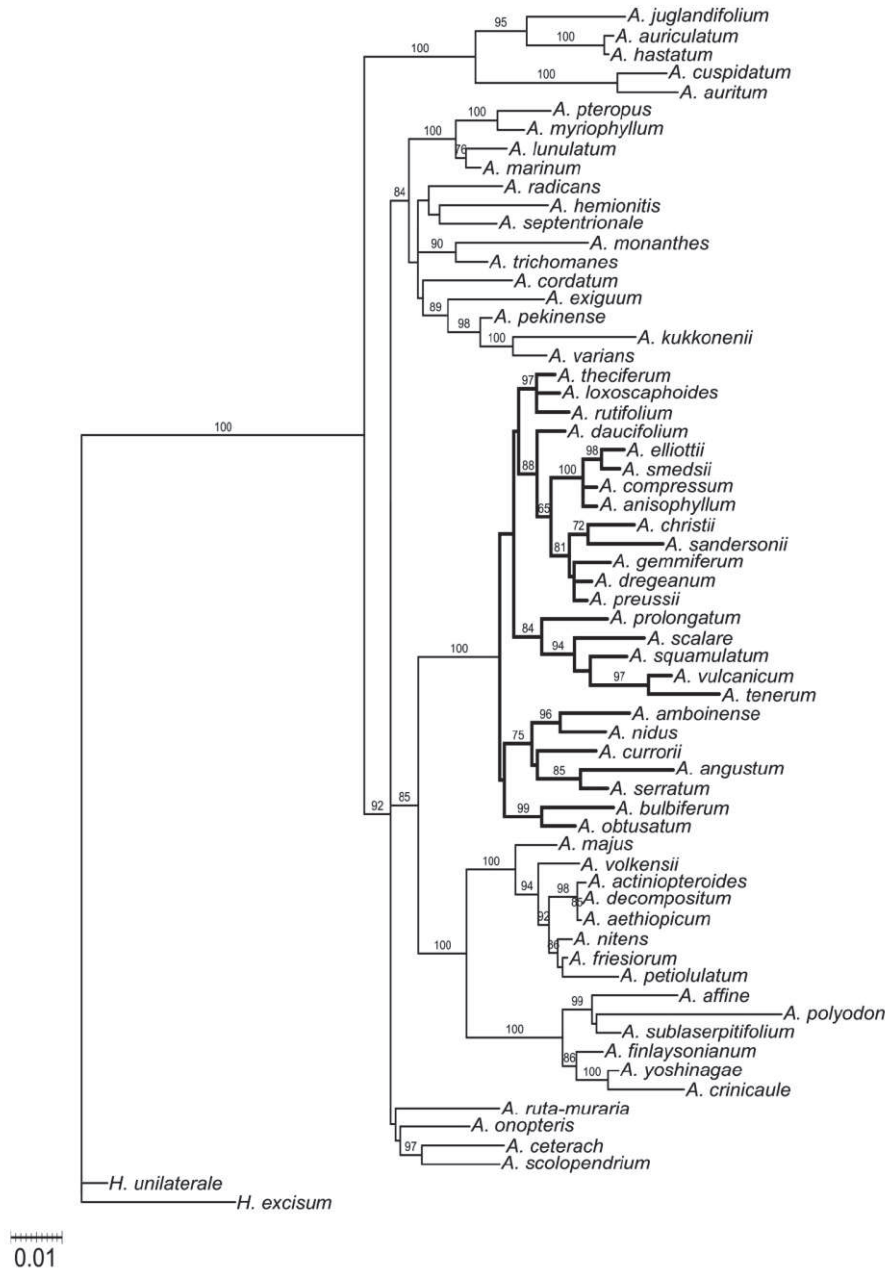


Figure 5.5: Maximum likelihood phylogeny based on 64 rbcL sequences. Bootstrap values higher than 70% are noted above branches. All taxa with HCWTs are grouped in a monophyletic clade indicated with thicker branch widths.

5.4 DISCUSSION

Structure of HCWTs

Morphologically similar velamen cells in orchids differ from *Asplenium* cortex cells in many aspects. Firstly, mature velamen cells are dead whereas *Asplenium* cortex cells remain alive. Secondly, velamen cells develop from a protoderm that becomes multilayered and then differentiates, which is not the case in *Asplenium*, where the protoderm remains unilayered and forms the rhizodermis. HCWTs develop close to the apical regions after cellular expansion has ceased and showed considerable uniformity of design. Their pattern consists of helical, regularly bifurcating and anastomosing strands. The strands on transverse walls were oriented either in parallel bands or in a random pattern, as shown by laser scanning confocal microscopy. In contrast to Orchidaceae, where different patterns have been described for different species (Sanford & Adanlawo, 1973), no significant differences in HCWT-patterns were observed between *Asplenium*-species. Because strands are unpaired on opposite sides of a common cell wall, as shown by transmission electron microscopy (Figure 5.3A), thickenings probably develop independently in adjacent cells.

According to Harris (2006) secondary cell walls are deposited on the primary cell walls after cell expansion has ceased. Because thickenings are deposited above the elongation zone of the root, thus after cell expansion has ceased, HCWTs in *Asplenium* are secondary cell wall deposits *sensu* Harris (2006). As reported by Esau (1965), the distinction between middle lamellae and primary cell walls becomes obscured during the extension growth of the cell. Especially in cells with extensive secondary cell walls, middle lamellae become extremely obscure. As a result, the primary cell walls of adjacent cells and their intercellular middle lamella appear as one unit, often incorrectly referred to as a middle lamella (Esau, 1965). Both cell walls of adjacent tracheids and of tracheids and adjacent xylem parenchyma cells showed that their intervening electron-dense zone includes the middle lamella and the primary cell wall of these cells.

Non-lignified secondary cell walls

Many studies have reported lignins in the secondary walls of the orchid velamen (e.g., Dycus & Knudson, 1957; Shushan, 1959; Morisset, 1964; Sanford & Adanlawo, 1973; Noel, 1974; Benzing *et al.*, 1983; Burr & Barthlott, 1991) and in tracheoidal cells (e.g. Dickison, 1973; Ter Welle, 1975). Most of these observations were based on the commonly used Wiesner-reaction with phloroglucinol/HCl. The negative result of our phloroglucinol test suggests that HCWTs in *Asplenium* are not lignified. However, typical red colour

reactions of phloroglucinol stainings could be masked in mature *Asplenium* root cortex cell walls because these are generally impregnated with brown pigments. Moreover, Müsel *et al.* (1997), using immunogold labeling, showed that lignins did occur in cell walls which were not stained by phloroglucinol. To overcome these constraints, we assessed the presence of lignins in the root cortex of *A. daucifolium* by a chemical analysis. Quantitative methods used to demonstrate lignins in hypolignified samples are often restricted by interference with other components (Hatfield *et al.*, 1999). Therefore, we applied a chemical reaction enabling the release of lignin monomers to provide chemical evidence of the presence of lignins. Thioacidolysis was preferred since it maintains the phenylpropane structure (Lapierre, 1993). In addition, thioacidolysis specifically disrupts the non-condensed inter-unit linkage which constitutes the main bonding pattern in Gymnosperm and Angiosperm lignins (Lapierre, 1993). Monolignol derivatives were not found after thioacidolysis of the outer root cortex of *A. daucifolium*, suggesting that lignin are absent. The few lignin monomers detected in the analysis of the entire root sample, must have been released by the included root xylem. The recovery yield was low (100 $\mu\text{mol/g}$ lignin) suggesting that tracheid cell walls are either weakly lignified, and/or that the number of xylary elements is low, and/or that their lignins contain a low proportion of alkyl-aryl linkages. In the entire root sample of *A. daucifolium*, the molar S/G ratio was very weak (0.13). This value is close to that obtained for flax xylem which contains fibre tracheids (Day *et al.*, 2009).

According to the classification of cell wall types of Harris (2006), HCWTs in Aspleniaceae are non-lignified secondary cell walls. In contrast to lignified secondary cell walls, which have been studied extensively, non-lignified secondary cell walls have not received much attention. One of the reasons being that most research groups working on cell walls often either focus only on non-lignified primary or on lignified secondary cell walls (Harris, 2006). Non-lignified secondary cell walls have been reported in thick-walled parenchyma cells in cotyledons, endosperm cells of many angiosperm seeds (Harris, 2005a), pollen tubes (Harris, 2005b), cotton seeds (Harris, 2005b), and fibers in *Gnetum* leaves (Tomlinson & Fisher, 2005). The secondary cell walls of transfer cells, which form ingrowths with a diverse morphology, are also non-lignified (Offler *et al.*, 2002; McCurdy *et al.*, 2008).

Instead of being lignified, all cell walls in mature Aspleniaceae roots, except for those of the endodermis and stele, are impregnated with brown pigments. A similar impregnation with brown pigments was found in secondary cell walls in elaters of the liverwort *Radula* (Kremer & Drinnan, 2002). Ogura (1972) referred to such brown

pigments in fern sclerenchyma as phlobaphenes, which can be detected by their yellow-brown colour, as suggested by Reeve (1951).

Presence of other major cell wall constituents

Immunolabeling indicated the presence of pectic homogalacturonan, cellulose, mannan and xyloglucan in HCWTs. Xylan, a typical component of xylary cell wall thickenings (Carafa *et al.*, 2005), was only detected in the tracheid walls of roots. CBM3a labeled all secondary walls and confirmed the positive calcofluor reaction showing the presence of crystalline cellulose in HCWTs. Our results indicate that wall component polymers of HCWTs are distinct from those in tracheidal walls. The LM6 arabinan antibody bound only to cell walls of the parenchymatous cells of the central cylinder and confirmed the findings of Leroux *et al.* (2007b), showing that this epitope was abundant in the same cell types in the vascular bundles of *Asplenium* petioles. LM21, a monoclonal antibody binding to a mannan-epitope, was the only antibody that bound both to the sclereids of the sclerenchyma sheath and to HCWTs. None of the antibodies used in this study labelled the compound middle lamellae of mature roots. In old roots all cell walls, including the HCWTs are impregnated with phlobaphenes and appear unlabelled suggesting that their impregnation probably blocks the access of probes to epitopes. A similar masking effect was reported by Marcus *et al.* (2008), where pectic homogalacturonan masked abundant sets of xyloglucan epitopes.

In summary, we can state that non-lignified HCWTs are rich in cellulose and pectins, and also contain xyloglucan and mannan. Due to the impregnation of HCWTs and of compound middle lamellae, conclusions regarding the absence of epitopes should be interpreted with care.

Taxonomical conclusions based on the distribution of HCWTs

As shown in the phylogenetic tree based on *rbcL* sequences, HCWTs are found in one clade (HCWT-clade) of mainly tropical taxa related or belonging to species complexes including, a.o., *A. nidus* and *A. theciferum*. We definitely found HCWTs in *A. nidus*, *A. anisophyllum*, *A. boltonii*, and *A. tenerum*, which were reported to lack HCTWs by Schneider (1997) leading to his alternative assessment of their taxonomic value. The four taxa we included in our analysis, for which Schneider (1997) reported HCWTs, also nest within the HCTW-clade. Thus, because HCWTs are restricted to a single clade with strong bootstrap support (*A. theciferum* to *A. obtusatum*) and can be considered a synapomorphy, we reject the hypothesis of parallel evolution of HCWTs in Aspleniaceae as suggested by Schneider (1997).

HCWTs are absent in the *Hymenasplenium* species studied, as well as in several temperate taxa such as *A. scolopendrium*, *A. trichomanes*, and *A. septentrionale*. About 30% of the taxa in the HCWT-clade are strict epiphytes, another 30% grow either as epiphytes or on rocks, and a minority is strictly terrestrial. However, taxa such as *A. anisophyllum*, *A. bulbiferum*, *A. compressum*, *A. elliotii*, *A. gemmiferum*, *A. loxoscaphoides*, *A. obtusatum*, *A. prolongatum*, and *A. sertularioides*, are only exceptionally epiphytic. Only few other strict epiphytes, such as *A. affine*, *A. juglandifolium*, and *A. pteropus* are present in some of the other major clades which contain a majority of epilitic or terrestrial taxa. Thus the occurrence of HCWTs is not restricted to ferns with an epiphytic lifestyle. Similarly, among orchids, the analogous ‘velamen’ covers roots of most epiphytes, but also occurs in some terrestrial orchids. We found strong bootstrap support for a sister relationship between the HCWT clade and a clade including *A. aethiopicum* (*A. majus* to *A. crinicaule*). A very similar topology, with less support but based on more genes, was presented by Schuettpelz *et al.* (2008). Our molecular tree shows some minor topological differences between the larger clusters in comparison to the one published by Schneider *et al.* (2004). However, essentially the same species groups are present, and the minor differences may be due to the inclusion of trnL sequences by Schneider *et al.* (2004).

Possible function(s) of HCWTs

HCWTs strengthen primary cell walls and may prevent the collapse of dehydrated or dead outer root cortex cells, stabilizing the root cortex mechanically as suggested by Schneider (1996). They may be an adaption to water stress which might otherwise result in the collapse of unstabilized cells. Another possible function of HCWT-supported outer root cortex tissue is the formation of a boundary layer reducing root transpiration in aerial roots, as suggested for the orchid velamen by Benzing *et al.* (1982). For species growing on substrates with a low nutrient content, HCWT-supported sponge-like cortex cells may allow a more efficient uptake and storage of nutrient-rich water.

5.5 CONCLUSIONS

This is the first report of non-lignified secondary cell walls in ferns. The abundance of cellulose, pectic homogalacturonan, mannan, and xyloglucan in HCWTs was demonstrated. As ferns are only of minor economic importance, information about their cell wall architecture is fragmentary. However, many recent publications (Sørensen *et al.*, 2008; Knox, 2008; Popper, 2008; Sørensen *et al.*, 2010; Popper & Tuohy, 2010) stressed that large-scale surveying of cell walls across the plant kingdom may yield valuable new data

that contribute to our knowledge of cell wall evolution. Moreover, studies of the cell walls of early land plants will deepen our understanding of the roles of plant cell walls as well as their molecular diversity and architecture (Stebbins, 1992). The occurrence of HCWTs in Aspleniaceae is taxonomically significant as we showed that taxa possessing HCWTs are grouped in one, strongly supported, monophyletic clade, leading us to reject the hypothesis of parallel evolution suggested by Schneider (1997). Where present, HCWTs may stabilise the cortex tissue mechanically, allowing a more efficient uptake of water and nutrients, preventing desiccation of the vascular stele, and act as a boundary layer increasing the diffusive pathway towards the atmosphere. The presence of HCWTs may be advantageous for epiphytic ferns, however, HCWTs in Aspleniaceae are not restricted to epiphytic species, and not all epiphytes contain them.

5.6 ACKNOWLEDGEMENTS

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5.7 REFERENCES

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Appendix. List of material used for the molecular analysis with voucher information and Genbank accession numbers.

- A. actiniopteroides*: Tanzania, South East of Mount Kilimanjaro: Viane 7583.
- A. aethiopicum*: Kenya, Aberdare National Park, Karura Falls: Viane 7377.
- A. affine*: Réunion, Forêt de Bébour, 6 km N of Rivière des Marsouins, c. 1550 m alt.: Viane 8228.
- A. amboinense*: Cultivated at Kebun Raya Bali.: sn.
- A. angustum*: Genbank, AY300106.
- A. anisophyllum*: Zimbabwe, Bvumba Mts., Cloudlands, c. 1590 m alt.: Viane 8775.
- A. auriculatum*: Puerto Rico, Sierra de Luquillo, Rio Grande, Mt Britton trail, c. 755 m alt.: Viane 10602.
- A. auritum*: Puerto Rico, c. 4 km due W of Maricao, c. 335 m alt.: Viane 10628.
- A. bulbiferum*: Genbank, AY283226.
- A. ceterach*: India, Tangling Village, Pawari, Peo, Kinnaur: Harris Chandra Pande 107295.
- A. christii*: Kenya, Western Prov., SE of Kakamega, c. 1750 m alt.: Viane 7453.
- A. compressum*: St. Helena, Southern slopes of Diana Peak, c. 615 m alt.: Viane 8852.

- A. cordatum*: Namibia, 7 km SE of Otjiwarongo, c. 1470 m alt.: Van den heede 817.
A. crinicaule: Myanmar, Kachin State, c. 1165 m alt.: Viane 9682b.
A. currorii: Botanical Garden Ghent University, Ivory Coast: 79-2824.
A. cuspidatum: Venezuela, Trujillo, c. 16 km SW of Bocono, c. 1865 m alt.: Viane 10112.
A. daucifolium: Réunion, Forêt de Bélouve, c. 1548 m alt. : Viane 8220.
A. decompositum: Tanzania, SE of Mt. Kilimanjaro, c. 2700 m alt.: Viane 7688.
A. dregeanum: Cameroon, Manengouba Mts, 1970 m alt.: Viane 2563.
A. elliotii: Tanzania, SE of Mt. Kilimanjaro, c. 2750 m alt.: Viane 7685.
A. exiguum: China, Yunnan, Dêqên County, c. 2100 m alt.: Viane 9341.
A. finlaysonianum: Myanmar, Kachin State, ENE of Puta-O, c.750 m alt.: Viane 9708.
A. friesiorum: Zimbabwe, Nyazengu Priv. Nat. Res., c. 1950 m alt.: Viane 8752.
A. gemmiferum: Genbank, AY300117.
A. hastatum: Venezuela, Mérida, c. 2090 m alt.: Viane 10182c.
A. hemionitis: Spain, Canary Islands, Gran Canaria, c. 400 m alt.: Viane 6679.
A. petiolulatum: Réunion, SE of Dos d'Ane, c. 1150 m alt.: Viane 8423.
A. juglandifolium: Puerto Rico, Cordillera Central, SE part of Toro Negro forest, c. 950 m alt.: Viane 10667.
A. kukkonenii: China, Yunnan, hills E of Zhongdian, c. 3410 m alt.: Viane 10784.
A. loxoscaphoides: Tanzania, S. of Arusha National Park (Mt. Meru), c. 1780 m alt.: Viane 7549.
A. lunulatum: Rep. South Africa, Eastern Cape, Amatole Range, Xolora Hills, c. 1450 m alt.: Viane 6558.
A. majus: Kenya, Mt. Elgon Nat. Park, Erica arborea zone, c 3470 m alt.: Viane 7427.
A. marinum: Genbank, AF240647.
A. monanthes: Kenya, Mt. Aberdare, Chania Falls, c. 2950 m alt.: Viane 7368.
A. myriophyllum: Venezuela, Sierra de la Culata, c. 3075 m alt.: Viane 10147.
A. nitens: Réunion, Forêt de Bélouve, c. 1548 m alt.: Viane 8203.
A. obtusatum: Genbank, AY283232.
A. onopteris: Spain, La Palma, SE of Llano Negro, c. 1270 m alt.: Viane 8094.
A. pekinense: China, Beijing, Xiang Shan: Viane 10031.
A. polyodon: Réunion, NW exposed slope of "Bras de la Plaine", c. 615 m alt.: Viane 8545.
A. preussii: Zimbabwe, Chirinda forest, SE of Mt. Selinda, c. 1150 m alt.: Viane 8798.
A. prolongatum: Myanmar, KachinState, ENE of Puta-O, c. 965 m alt.: Viane 9657.
A. pteropus: Venezuela, Sierra de la Culata, Las Cuadras, c. 2370 m alt.: Viane 10156.
A. radicans: Venezuela, Sierra de la Culata, rivulet forest along road to Las Cuadras, c. 2345 m alt.: Viane 10161.
A. ruta-muraria: Italy, Mt. Lessini, c. 1715 m alt.: Viane 9553.
A. rutifolium: Zimbabwe, Bvumba Mts, Bunga Forest, c. 1634 m alt.: Bellefroid 245.
A. sandersonii: Tanzania, SE of Mt. Kilimanjaro, 2250 m alt.: Viane 7719a.
A. scalare: Malaysia: State of Perak; Bukit Larut, 940 m alt. on the right side to the top hill: KH155.
A. scolopendrium: Belgium, E. Flanders, Ghent, Malmaz ruins, c. 6 m alt.: Viane 10971.
A. septentrionale: France, Savoie, SE of Albertville, NW of Bar Cenisio, c. 2010 m alt.: Viane 9578.
A. serratum: Puerto Rico, Sierra de Luquillo, Rio Grande, c. 390 m alt.: Viane 10607.
A. smedsii: Tanzania, SE of Mt. Kilimanjaro, 2470 m alt.: Viane 7707.
A. squamulatum: Indonesia: Jambi Province; Kerinci-Seblat National Park; Bukit Tapan, 918 m alt.: KH94.
A. sublaserpitiifolium: Myanmar, Kachin State, 450 m alt.: Viane 9714.
A. tenerum: Indonesia: Nusa Tenggara Barat – Mataram: Mt. Rinjani, 550 m alt.: KH174.
A. theciferum: Zimbabwe, Bvumba Mts, Bunga Forest, c. 1610 m alt.: Bellefroid 238.
A. trichomanes: India: Niti Village, Joshimath Pawari, Chamali, Uttaranchal: Harris Chandra Pande 106448.
A. varians: China, Yunnan, forested hillside W of Kang Pu, c. 1850 m alt.: VIANE 9348.
A. volkensii: Tanzania, SE of Mt. Kilimanjaro, c. 2700 m alt.: VIANE 7689.
A. vulcanicum: Indonesia: West Java Province; Mt. Gede-Pangrango National Parks, 1550 m alt.: KH136.
A. yoshinagae: China, Xizang (Tibet), Nyingtri distr., c. 2200 m alt.: VIANE 10879.
H. excisum: Indonesia: Jambi province; Kerinci-Seblat National Park; Mt. Kerinci, 1675 m alt.: KH17.
H. unilaterale: Réunion, Forêt de Bébour, c. 900 m alt.: VIANE 8334.

6

Ultrastructure and composition of cell wall appositions in the roots of *Asplenium* (Polypodiales)

This chapter is a modified version of:

Leroux O, Leroux F, Bagniewska-Zadworna A, Knox JP, Claeys M, Bals S, Viane RLL. Ultrastructure and composition of cell wall appositions in the roots of *Asplenium* (Polypodiales) (submitted).

Summary

Cell wall appositions (CWAs), formed through deposition of extra wall material at the contact site with microbial organisms, are an integral part of the response of plants to microbial challenge. Detailed histological studies of CWAs in fern roots do not exist. Using light and electron microscopy we examined the (ultra)structure of CWAs in the outer layers of roots of *Asplenium* species. All cell walls studded with CWAs were impregnated with yellow-brown pigments. CWAs had different shapes, ranging from warts to elongated branched structures, as observed with scanning and transmission electron microscopy. Ultrastructural study further showed that infecting fungi grow intramurally and that they are immobilized by CWAs when attempting to penetrate intracellularly. Immunolabelling experiments using monoclonal antibodies indicated pectic homogalacturonan, xyloglucan, mannan and cellulose in the CWAs, but tests for lignins and callose were negative. We conclude that these appositions are defense-related structures made of a non-lignified polysaccharide matrix on which phenolic compounds are deposited in order to create a barrier protecting the root against infections.

6.1 INTRODUCTION

The cell wall is the interface for some of the interactions between plants and a wide variety of micro-organisms, ranging from bacteria to Oomycetes, Chytridiomycetes, Zygomycetes and true fungi (Parniske, 2000). Cell wall appositions (CWAs) are warty to narrowly elongated structures formed at, and continuous with, the inner cell wall surface through deposition of extra wall material at the site of contact with microbes. They are an integral part of the response of plants to microbial challenge (Aist, 1976; Bestwick *et al.*, 1995; Brown *et al.*, 1998).

According to Aist (1976), localised cell wall thickenings were described for the first time by de Bary in 1863 as a reaction to fungal penetration. Since then, numerous studies have been published, referring to these structures as papillae (e.g., Bordallo *et al.*, 2002; Brown *et al.*, 1998; Davey *et al.*, 2009; Oriani and Scatena, 2007; Schmelzer 2002), callosities (Young, 1926), lignitubers (e.g., Fellows, 1928; Griffiths and Lim, 1964; Griffiths, 1970; Smith 1900), 'röhrentüpfen' (Burgeff, 1938; Boullard, 1957), or cell wall appositions (e.g., Beswetherick and Bishop, 1993; Greenshields *et al.*, 2004). These wall-like structures have also been related to plant resistance to the attack of other micro-organisms such as viruses (Allison *et al.*, 1974; Kim and Fulton, 1973) and bacteria (Aist, 1976; Sherwood and Vance, 1976; Siranidou *et al.*, 2002; Yedidia *et al.*, 1999). As the occurrence of CWAs is often associated with an increased penetration failure, the formation of these structures is considered to be an important plant defence mechanism (Aist, 1976). They are formed during a non-specific reaction, and more specifically during non-host, incompatible or compatible interactions with non-virulent or virulent micro-organisms (Hardham *et al.*, 2008).

Many studies, mainly based on histological and chemical analysis, demonstrated the heterogeneous composition of CWAs. In most cases, lignin (e.g., Fellows, 1928; Griffiths, 1964; Griffiths, 1970; Sherwood and Vance, 1976) and callose (e.g., Aist, 1976; Cordier *et al.*, 1998; Mims and Vaillancourt, 2002; Rey *et al.*, 1998; Roussel *et al.*, 1999; Sherwood and Vance, 1976; Smith and Peterson, 1985) were identified as main components, but cellulose, chitin, gums, silicon, suberin and proteins have also been reported (Aist, 1976).

Only few studies on cell wall appositions in ferns have been published. In a study on fern root hairs and rhizoids, Pearson (1969) mentioned that the inner surface of the outermost cortical walls is often studded with brown tubercles and that in extreme cases the cortex, epidermis and root hairs are full of such structures. CWAs have also been reported in *Adiantum* leaves in response to infection by the ascomycete *Botrytis cinerea* (Archer and Cole, 1986).

Many roots are able to form symbiotic relationships with micro-organisms (Linderman, 1996). The most complex are those involving nodule formation in association with bacteria or actinomycetes (Golotte *et al.*, 1996). However, the most common are mycorrhizas resulting from the invasion of root tissues by various fungi (Bonfante and Anca 2009). Mycorrhizas are commonly divided into arbuscular mycorrhizas (AM) and ectomycorrhizas (EM). AM are the most widespread, characterised by hyphae penetrating individual cells in the root. EM, on the other hand, mainly occur in gymnosperm and angiosperm roots and do not penetrate intracellularly (Harley and Smith, 1983). Mycorrhizal associations have been investigated in the roots of many ferns and lycophytes and the majority was found to be mycorrhizal with AM associations (for a review, see Burgeff, 1938; Boullard, 1957; Brundrett, 2008). In leptosporangiate ferns, Paris-type arbuscular mycorrhizal (AM) structures were observed in most cases (Smith and Smith, 1997; Zhang *et al.*, 2004). These are characterized by the absence of an intercellular phase of hyphal growth, the presence of intracellular hyphal coils, and/or arbuscules as intercalary structures on the coils (Smith and Smith, 1997). However, many fern species with fine roots and long root hairs have limited or inconsistent mycorrhizal colonisation. These facultative mycorrhizal associations are most common for leptosporangiate ferns, including Aspleniaceae (Brundrett, 2002). Moreover, epiphytic and epilythic, as well as aquatic ferns are less likely to be mycorrhizal, as they generally grow in habitats where mycorrhizal fungi may not be present (Brundrett, 2002).

The family Aspleniaceae is one of the most species-rich among leptosporangiate ferns with over 720 species (Kramer and Viane, 1990), showing remarkable ecological and systematic diversity. Plants are either terrestrial, epilithic or epiphytic, and occur from sea level to over 4000 m altitude. Most taxa are found in tropical montane rainforests and south-temperate regions, though some groups are adapted to xeric conditions.

Here we report a study of the structure and composition of CWAs in several *Asplenium* species, based on light microscopy, laser scanning confocal microscopy, and scanning and transmission electron microscopy.

6.2 MATERIAL AND METHODS

6.2.1 Material

Fresh roots of the following species were obtained from our living collection kept at the Ghent University Botanical Garden (collected by R. Viane (RV), E. Bellefroid (EB) and Caroline Van den heede (CV)): *A. abyssinicum* (RV11186 – Uganda), *A. centrafricanum* (EB392 – Uganda), *A. ceterach* (CV250b – Cyprus), *A. crinicaule* (RV11304 – China), *A. currorii* (RV sn – Ivory Coast), *A. elliottii* (RV7685 – Republic of South Africa, RV7708 – Tanzania), *A. exiguum* (RV10813 – China), *A. formosum* (RV10620 – Puerto Rico), *A. hastatum* (RV10239 – Venezuela), *A. hemionitis* (nn – unknown), *A. juglandifolium* (RV10667 – Puerto Rico), *A. monanthes* (RV11106 – Mexico), *A. nidus* (RV11260 – China), *A. radicans* (RV10162 – Venezuela, RV10639 – Puerto Rico), *A. rutifolium* (RV6377c – Republic of South Africa), *A. sandersonii* (RV7719 – Tanzania), *A. scolopendrium* (RV10971 – Belgium), *A. serra* (RV10222 – Venezuela), *A. tenerum* (RV9043 – Indonesia), *A. theciferum* (EB238 – Zimbabwe, EB323 – Zimbabwe), *A. trichomanes* (RV6217 – Belgium), *A. volkensii* (RV7313 – Kenya, RV11124 – Uganda). One sample was fixed in the field: *A. sertularioides* (EB357 – Uganda).

6.2.2 Histology

For light microscopy, roots were fixed in FAA (90% v/v ethanol 50%, 5% v/v acetic acid and 5% v/v commercial formalin). Samples were dehydrated in an ethanol series, embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) and sectioned following Leroux *et al.* (2007). Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200. Phloroglucinol/HCl and Maüle staining was performed following Leroux *et al.* (2010).

6.2.3 Immunohistochemistry

For transverse sections, roots of *A. elliottii* were clamped in between two sheaths of Parafilm M (Pechiney Plastic Packaging Inc, Neenah, WI, USA) and sectioned using a single edged razor blade. A range of probes directed against cell wall polysaccharides was used to check the presence of some major cell wall polymers in CWAs. These included the anti-homogalacturonan monoclonal antibodies JIM5, JIM7 and LM19 (Clausen *et al.*, 2003; Verhertbruggen *et al.*, 2009); anti-galactan antibody LM5 (Jones *et al.*, 1997), anti-arabinan antibody LM6 (Willats *et al.*, 1998), anti-xylan antibody LM11 (McCartney *et al.*, 2005), anti-xyloglucan antibody LM15 (Marcus *et al.*, 2008), anti-(galacto)(gluco)mannan LM21 (Marcus *et al.*, 2010), the his-tagged carbohydrate binding

module CBM3a directed to crystalline cellulose (Blake *et al.*, 2006) and the Biosupplies 400-2 anti-callose antibody (Meikle *et al.*, 1991). Immunolabeling was performed as described elsewhere (Blake *et al.*, 2006; Leroux *et al.*, 2010; Meikle *et al.*, 1991). Images were captured using a laser scanning confocal microscope (Nikon e-C1 mounted on a Nikon TE2000). After exciting the staining signal at 488 nm using an Argon laser and detecting the fluorescence emission with a 540 nm long pass filter, photos were made at a X/Y resolution of 512x512 pixels. Z-stacks were made every 0.5 μm and combined in one X/Y image.

6.2.4 Scanning electron microscopy

Longitudinal hand-cut sections of roots were fixed in FAA and processed following Leroux *et al.* (2010). Samples were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

6.2.5 Transmission electron microscopy

Root cortex tissue was dissected in order to produce blocks measuring approximately 5 mm length at all sides. They were fixed with w/v 4% paraformaldehyde and 2.5% v/v glutaraldehyde in a cacodylate buffer 0.1 M pH 6.9 for 24 h at 4°C, washed in the same buffer and dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100% alcohol/LRWhite resin (1:1) at 4°C overnight, brought to 100% alcohol/LRWhite (1:2) for 8 h (4°C), and transferred to 100% LRWhite resin and left overnight at 4°C. Polymerization was performed at 60°C for 16 h in a flat embedding mold. 100-nm-thick sections were made using a Reichert ultracut S Ultramicrotome. Thin bar 600-mesh copper grids were used without support film. Sections were stained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 10 min in lead citrate stain at 20°C. A thin layer of amorphous carbon was evaporated to increase stability under the electron beam. The grids were examined with a FEI Tecnai F20 operating at 200kV.

6.3 RESULTS

The general structure of the roots studied did not differ from that commonly found in most leptosporangiate ferns (Ogura, 1972). Cell walls of the inner cortex are heavily thickened whereas cells of the outer cortex and epidermis remain thin-walled (Fig. 6.1a, b). In many *Asplenium* species helical cell wall thickenings are deposited on the inner cell wall surface of the outer root cortex (Leroux *et al.*, 2010; Fig. 6.1c). Aspleniaceae are also characterised by the absence of intercellular spaces in all root tissues. After observing

CWAs in the epidermis and outer cortical cells of *A. elliotii* (Fig. 6.1a, b) we also examined other *Asplenium* species kept in greenhouses at the Ghent University Botanical Garden. We found similar structures in the roots of *A. abyssinicum*, *A. centrafricanum*, *A. ceterach*, *A. crinicaule*, *A. currorii*, *A. elliotii*, *A. exiguum*, *A. formosum*, *A. hastatum*, *A. hemionitis*, *A. juglandifolium*, *A. monanthes*, *A. nidus*, *A. radicans*, *A. rutifolium*, *A. sandersonii*, *A. scolopendrium*, *A. serra*, *A. tenerum*, *A. theciferum*, *A. trichomanes*, and *A. volkensii*. We examined roots of *A. sertularioides* which were fixed in the field and also observed CWAs.

CWAs are large enough to be visible at the light microscopic level of resolution (Fig. 6.1b), and appeared primarily on the outer tangential cell walls of the outer cortical and epidermal cells. They varied in size and shape, ranging from wart-like to elongated structures with a length up to 15 μm , with some of them being branched (Fig. 6.1b). They mainly occurred in the epidermal and in the outer cortex of 'old' roots with fully differentiated metaxylem tracheids, a sclerenchyma sheath composed of several cell layers of sclereids, and cortex cell walls impregnated with yellow-brown pigments (Fig. 6.1a). We never found CWAs in the apical regions of the roots nor in young lateral roots. All cell walls with CWAs were impregnated with yellow-brown pigments as shown in figure 6.1a, b. In roots with extremely abundant CWAs, all outer cortical and epidermal cells were dead and collapsed. We observed few signs of fungal colonisation. In only a few cases, filamentous fungal-like structures with a diameter ranging from 0.2 to 0.4 μm were seen in the outer cell layers of the root (Fig. 6.1d).

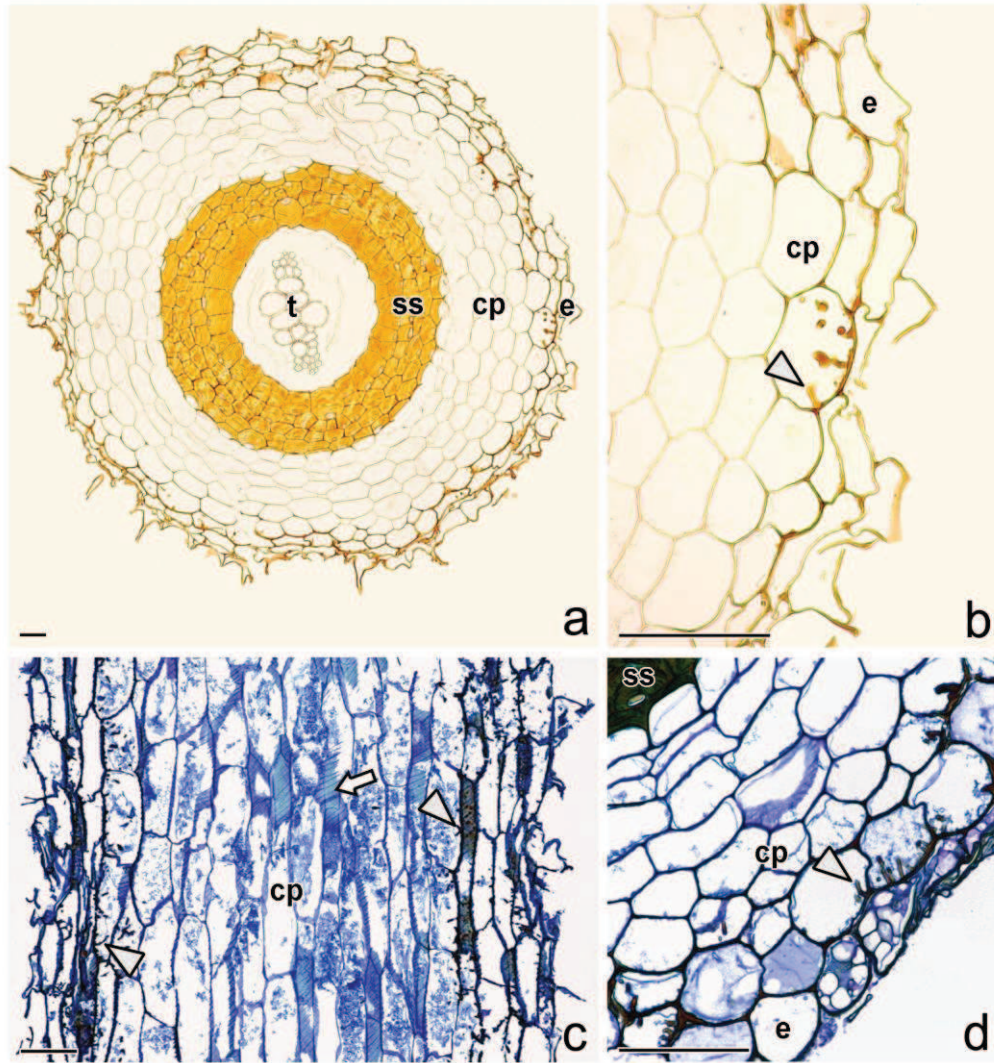


Figure 6.1: Light micrographs of *A. elliotii* root sections. (a) unstained cross section showing the epidermis (e), cortical parenchyma (cp), sclerenchyma sheath (ss) and the central vascular bundle with tracheids (t). Note that both the cell walls of the sclerenchyma sheath and all cell walls bearing cell wall appositions are infused with yellow-brown pigments. (b) Detail of (a) showing cell wall appositions (arrowhead). (c) Longitudinal section stained with toluidine blue showing CWAs (arrowheads) in the outer cell layers of the root as well as helical cell wall thickenings (arrow) in the cortical parenchyma. (d) Cross section stained with toluidine blue showing vesicle-like fungal structures in the epidermis. CWAs are indicated with arrowheads. Scale bars: 100 µm.

We studied epidermal and outer cortical cells using scanning electron microscopy and confirmed that epidermal and cortical cell walls were studded with numerous wart-like (Fig. 6.2a) or elongated (Fig. 6.2b, c) and often branched (Fig. 6.2c) CWAs. We found CWAs both in between and on top of the helical cell wall thickenings (Fig. 6.2a). The surface of the CWAs was rather granular. Cells with CWAs (Fig. 6.2 a, c, d) frequently contained fungal-like structures. However, these are not necessarily responsible for the CWAs and some might be actinomycetes.

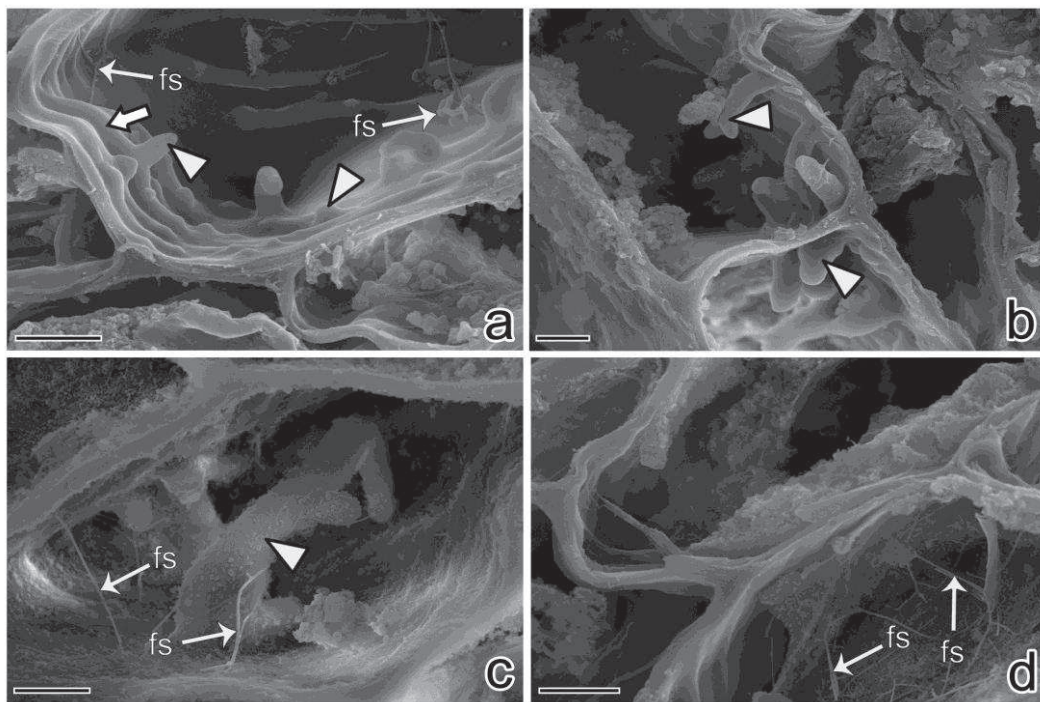


Figure 6.2: Scanning electron micrographs of *A. Elliottii* roots. (a) inner cell wall surface of a root cortex cell showing helical cell wall thickenings (arrow), numerous wart-like and finger-like CWAs (arrowheads) as well as fungal-like structures (fs). (b) CWAs (arrowheads) predominantly occur on the inner surface of outer tangential cell walls. (c) Image showing CWAs (arrowhead) co-occurring with fungal-like structures (fs). (d) Fungal-like structures (fs) in the epidermis. Scale bars: 6 μ m.

Transmission electron microscopy provided important additional information as it showed that CWAs were always associated with microbial colonization. Microbes were found either intracellularly, or in the cell walls and most of the CWAs (Fig. 6.3a). In some cells fungal structures partially penetrated the cell walls (Fig. 6.3a). The diameter of the penetration pegs ranged from 0.1 to 0.4 μm . We did not observe dolipore septa nor any other morphological characters that could help identifying the penetrating microbe. Varying amounts of host wall material were deposited between the host plasma membrane and cell wall at the site of potential penetration. The penetrating fungus appeared to be immobilised by the CWAs except where a host cell was subjected to many simultaneous penetrations. Most of the observed cells were dead judging from the remnants of a partly disintegrated plasma membrane. Microbial entrapments are most apparent in cross sections of finger-like CWAs (Fig. 6.3b). The deposited material was often stratified (Fig. 6.3b), suggesting that additional cell wall material was laid down as the microbe was trying to escape the CWA. Most CWAs were characterised by a distinct margin with a lower electron density (Fig. 6.3c). In some cases we noticed deposits along a large portion of the cell wall covering both the primary cell walls and the helical cell wall thickenings (Fig. 6.3d). Cell wall deposition seemed not to be restricted to sites of attempts of fungal penetration but also appeared to occur in uninfected cells adjacent to infected cells. However, serial sectioning of roots was not performed, and consequently signs of penetrating microbes are not necessarily visible in a single thin section through a root cortex cell. Next to the formation of CWAs we also observed an increasing electron density of the root cortical cell walls, especially in comparison with the helical cell wall thickenings (Fig. 6.3d), except in older roots where the complete cell wall, including the helical cell wall thickenings, is impregnated with these electron-dense deposits (data not shown). Most of the microbes were found inside the cell wall (Fig. 6.3e) including the middle lamella region, suggesting that fungal migration occurs primarily via the cell wall. Encased hyphae appeared to be dead in some cases as they were devoid of their protoplasm (Fig. 6.3e).

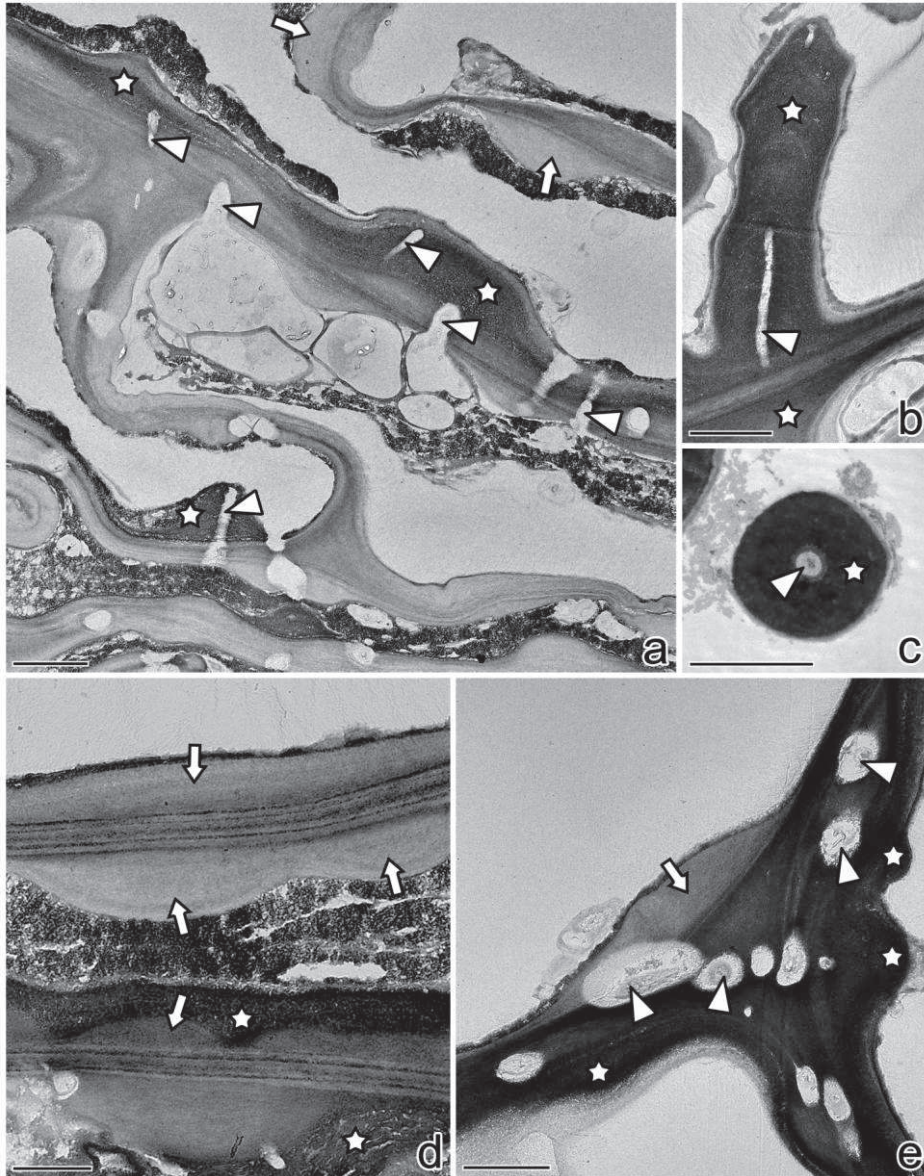


Figure 6.3: Transmission electron micrographs showing the presence of fungi within cell walls and CWAs. (a) Dead and partly compressed cortical parenchyma showing fungal structures that partly penetrated cell walls (arrowheads). (b) Longitudinal section through an elongated CWA showing the encased microbe. (c) Transverse section through an elongated CWA showing cell wall material deposited on a penetrating microbe. Note that less electron-dense outer layer of the CWA. (d) Image showing deposits of cell wall material covering some helical cell wall thickenings (arrows). (e) Cell wall junction in the root cortex showing numerous microbes within the cell walls as well as some small CWAs (asterisk). Infecting microbes indicated with arrowheads, helical cell wall thickenings with arrows and CWAs with asterisks. Scale bars: 2 μm .

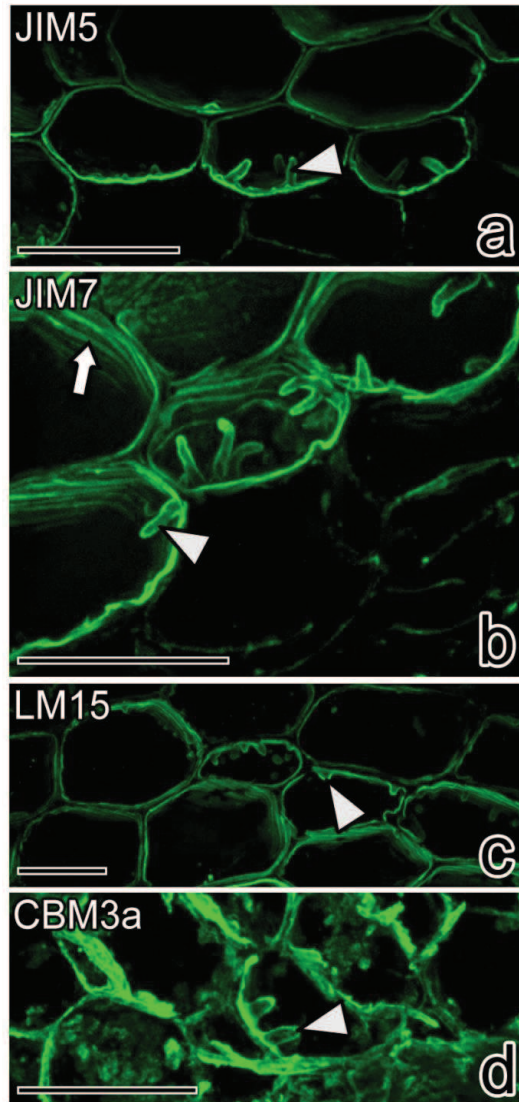


Figure 6.4: Indirect immunofluorescence labelling of cell wall polymers on *A. elliotii* root cross sections. (a,b) Antibodies directed against pectic homogalacturonan with low (JIM5) and high (JIM7) levels of methyl-esterification bind to CWAs as well as to the helical cell wall thickenings. (c) LM15 recognising the XGGG-motif of the hemicellulose xyloglucan binds to CWAs and helical cell wall thickenings in similar manners as JIM5 and JIM7. (d) CBM3a, a carbohydrate-binding module directed to crystalline cellulose labels CWAs and associated cell walls. All images are reconstructions of confocal optical sections. Scale bars: 30 μm .

To examine the composition of the cell wall appositions, we used a range of monoclonal antibodies (mAbs) with specificities for cell wall polymers including pectic homogalacturonan (JIM5, JIM7, LM19), RG-I-related galactan (LM5), RG-I-related pectic arabinan (LM6), xylan (LM11), xyloglucan (LM15), (galacto)(gluco)mannan (LM21), and callose (Biosupplies 400-2) as well as a carbohydrate-binding module recognizing crystalline cellulose (CBM3a). The mAbs JIM5, JIM7 and LM19, which all bind to

homogalacturonan with different levels of esterification, clearly labelled the CWAs as shown for JIM5 and JIM7 in Fig. 6.4a, b. The LM15 mAb, raised against the XXXG-motif of xyloglucan, the mannan LM21 mAb, and the CBM3a probe with specificity for crystalline cellulose, bound to the CWAs and associated cell walls in similar ways as the anti-pectin mAbs (Fig. 6.4c,d). MABs with specificity for (1→4)-β-D-galactan (LM5), (1→5)-α-D-arabinan (LM6), and xylans (LM11) did not bind to the CWAs nor to any of the root cortical cell walls. Callose, visualized using a mAb recognizing (1→3)-β-D-glucan, was not detected in the CWAs. Both the phloroglucinol and Maüle tests failed to stain the CWAs.

6.4 DISCUSSION

The absence of a cuticle and the abrasion of external cell layers during growth expose roots to a variety of potentially pathogenic micro-organisms. Therefore, they have developed many types of physical and chemical defense strategies including the deposition of cell wall material (CWAs), cell wall fortification through infusion with phenolics, and the production of anti-microbial products (Lucas, 2002).

CWAs have never been reported for Aspleniaceae and in only a few other ferns similar structures have been described (Archer and Cole, 1986; Boullard, 1957). CWAs occurred in all species investigated and collected at different localities, either from the wild or cultivated at the botanical garden, outdoors or in greenhouses. We found CWAs mainly in older roots close to the rhizome, and more rarely in young roots. However, the impregnation with yellowish-brown phenolic compounds of cell walls of the outer cells in young roots might constitute an early reaction to infection caused by the intercellular migration of a microbe. As we also found CWAs in roots fixed in the field, the occurrence of CWAs is widespread and not limited to infections induced during cultivation.

CWAs are produced in the epidermal and hypodermal cells and their associated cell walls are impregnated with yellow-brown pigments. CWAs were primarily found on the inner surface of outer tangential cell walls and their shape was variable, ranging from wart-like deposits to elongated and branched structures. Using transmission electron microscopy we showed that microbes, migrating through the cell wall, were sheathed in cell wall material in order to prevent intracellular penetration. Moreover, CWAs were always associated with microbial colonisation.

It has been reported that CWAs occurring in different plants may be ultrastructurally and compositionally diverse (Aist, 1976). They generally consist of cell wall materials which are considered to contribute in cell wall reinforcement and

resistance (Gianinazzi-Pearson *et al.*, 1996). Callose (β -1,3-glucan) is the most commonly identified chemical constituent in CWAs (e.g. Aist and Williams, 1971; Sherwood and Vance, 1976; Skou, 1982). Most of these studies relied on aniline blue fluorescence for detection of callose. Here, we used an anti- β -1,3-glucan antibody and this epitope was not detected in CWAs of *A. elliotii*. Immunolabelling further indicated the presence of partially methyl-esterified pectic homogalacturonan, xyloglucan, mannan and crystalline cellulose in CWAs. These polymers were also found to occur in helical cell wall thickenings (Leroux *et al.*, 2010), suggesting that a similar mechanism could be responsible for both cell wall depositions. Lignins have been found in CWAs of many plants (e.g. Fellows, 1928; Griffiths and Lim, 1964; Griffiths, 1970; Sherwood and Vance, 1976; Yedidia *et al.*, 1999). Negative phloroglucinol/HCl and Maïle tests, however, suggested that CWAs in *Asplenium elliotii* are non-lignified. Leroux *et al.*, (2010) chemically traced lignins in the outer cortex tissue of *A. elliotii* by means of thioacidolysis. The root cortex of the samples that were studied contained CWAs. The fact that they found no evidence for the presence of lignins, confirmed our negative phloroglucinol and Maïle test results, and suggests the absence of lignins in CWAs of *A. elliotii*. All cell walls in *Asplenium* roots associated with CWAs were found to be impregnated with yellow-brown pigments. Similar components have been attributed to phenolic compounds (Albersheim *et al.*, 2010). Phenolic compounds such as condensed tannins and flavonoids can occur as constitutive molecules present in healthy plants, but can also be synthesised as a result of plant infection or wounding in order to increase the resistance to fungal enzymes as well as constituting a stronger physical barrier preventing fungal penetration (Codignola *et al.*, 1989; Yao *et al.*, 2007). Rapid synthesis of phenolic compounds and their subsequent polymerisation in the cell wall have been suggested to be a first plant defence response against penetration attempts by parasitic and pathogenic fungi (Aist, 1976; Matern *et al.*, 1995). Some of these phenolic compounds may possess anti-microbial properties (Soylu *et al.*, 2003) or could modify the host cell wall in a way to resist the action of lytic enzymes produced by the pathogen (Matern *et al.*, 1995). Phenolic impregnation can provide high rigidity and indigestibility to host cell walls when they are linked with cell wall carbohydrates such as hemicelluloses and pectins through peroxidase-mediated cross-linking (Fry, 1986). As the infusion of these components starts in the middle lamellae, this cell wall modification directly affects penetration capability of fungi penetrating between cells by dissolution of the middle lamella. However, Stafford (1988) reported that the infusion of walls with flavanols or condensed tannins may also result passively from the loss of integrity associated with cell aging or death.

CWAs have often been referred to as papillae, callosities and lignitubers (Aist, 1976; Fellows, 1928). Considering the heterogeneity of compounds found in many studied wall appositions, the terms ‘callosity’ and ‘lignitubers’ are not used in this study, as these names imply the presence of callose and lignin respectively, and evidence for the presence of these molecules was not found in the CWAs of *A. elliotii*. Naming these structures CWAs does not require any specificity on morphology nor composition. In many cases cell wall material was laid down on the cell wall adding only additional layers instead of forming lignituber or papilla-like structures.

Notwithstanding the fact that we primarily focussed on the structure and composition of CWAs, the identity of the microbe(s) responsible for these cell wall deposition remains unclear. Since the first description by de Bary in 1863 (Aist, 1976), CWAs have always been related to plant resistance against the attack of many micro-organisms. More than 100 years of research suggests that most plants in natural ecosystems are symbiotic with mycorrhizal fungi and/or fungal endophytes (Rodriguez *et al.*, 2009). AM were reported for *Asplenium scolopendrium*, *A. trichomanes*, *A. bulbiferum* and *A. ruta-muraria* (Hepden, 1960). During our study we found no clear evidence suggesting the presence of mycorrhizal associations. While mycorrhizal proliferation in epidermal and hypodermal layers, as well as in the central cylinder is restricted (Bonfante and Genre, 2008; Gianinazzi-Pearson *et al.*, 1996), we always detected CWAs in the outer layers of the root. Moreover, in the case of AM, roots of most plants also show remarkably little reaction at the cytological level to penetration hyphae and no significant modifications occur in associated cell walls (Gianinazzi-Pearson *et al.*, 1996). Finally, the absence of coils and arbuscules in association with the CWAs suggests that the infecting microbes are not mycorrhizal. The small diameter of the hyphae and the absence of dolipore septa indicate that a basidiomycete is probably not responsible for the formation of CWAs. CWAs, similar to the ones studied here, have more frequently been described in association with plant-pathogenic Ascomycota such as *Bipolaris* (Carlson and Jansson, 1991), *Botrytis* (Archer and Cole, 1986), *Colletotrichum* (Mims and Vaillancourt, 2002; Wharton *et al.*, 2001), *Erysiphe* (Smith, 1900; *Fusarium* (Beswetherick and Bishop, 1993; Griffiths and Lim, 1964; Smith and Peterson, 1985), *Leptosphaeria* (Roussel *et al.*, 1999), *Gaeumannomyces* (Yu *et al.*, 2010), *Ophiobolus* (Fellows, 1928), *Trichoderma* (Yedidia *et al.*, 1999), and *Verticillium* (Griffiths and Lim, 1964; Griffiths, 1970, Storey and Evans, 1987). In most cases, they penetrate plants by use of penetration pegs which are thin tip-growing cellular protuberances, generally less than 1 μm in diameter (Howard, 1997). Based on the absence of any mycorrhizal structures and the similarity between the CWAs caused by many ascomycetes and the ones studied here, it is likely that ascomycetes are

responsible for the CWAs in Aspleniaceae. It is possible that they are the result of infection by several different species in response to the mechanical force of penetration, and/or a chemical stimulus caused by local dissolution of the cell wall at penetration sites.

6.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The results of our study show that invading microbes are sheathed in CWAs to prevent intracellular colonisation. CWAs are composed of pectic homogalacturonan, xyloglucan, mannan and cellulose. Callose and lignins, which have been found in CWAs of many other species, were not detected but cell walls associated with CWAs were impregnated with yellow-brown phenolic compounds. Remaining questions for future research lie in determining the role of each of the constituents of CWAs as well as identifying which micro-organisms are responsible for these cell wall modifications.

Controlled experiments in which model ferns species (e.g., *Ceratopteris*), grown in sterile conditions, are inoculated with specific pathogens may shed more light on the early events during infection as well as on plant-pathogenic interactions in ferns in general.

6.6 ACKNOWLEDGEMENTS

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Part 3

A focus on fern cell walls

7

An antibody-based analysis of fern cell walls confirms cell wall diversity and taxonomic patterns in the plant kingdom

This chapter is a modified version of:

Leroux O., Sørensen I., Marcus S.E., Espeel M., Viane R.L.L., Willats W.G.T. and Knox J.P. An antibody-based analysis of fern cell walls confirms cell wall diversity and taxonomic patterns in the plant kingdom (unpublished results).

Summary

The cell walls of 76 fern species, including 36 representatives of *Asplenium* and *Hymenasplenium* from within the leptosporangiate family Aspleniaceae, have been analysed with 34 monoclonal antibodies using the comprehensive microarray molecular polymer profiling (CoMPP) technique. The analysis was supplemented by *in situ* immunofluorescence and tissue immunoprinting analyses with selected species. The results indicate that (1) many cell wall polysaccharide epitopes of more advanced taxa are widely distributed across the fern species, (2) the distribution patterns of some epitopes are taxonomically significant, and (3) some epitopes are associated with specific tissues, tissue-types or cell wall modifications. In several cases, such as the rhamnogalacturonan-I associated 1,5-arabinan and 1,4-galactan, the epitopes were widely present but in novel restricted patterns in relation to tissues and cell types. Xyloglucan, xylan and mannan epitopes were also widely detected. The CCRC-M1 fucosylated xyloglucan epitope displayed a clear taxonomic pattern in its absence from the Aspleniaceae and homosporous lycophytes. Xylan was detected in the primary cell walls in lycophytes and some ferns. The LM21 mannan epitope was specifically associated with sclerified cell walls throughout the fern samples and detected widely in primary cell walls only after enzymatic removal of pectic HG. Glycan epitopes of arabinogalactan-proteins displayed complementary patterns of taxonomic restriction. These observations are discussed in context with what is known about the location and functions of these cell wall polysaccharides in other plant groups.

7.1 INTRODUCTION

Cell walls are important cell components underpinning several aspects of plant growth and development. An understanding of the relationship between the fine structures and functions of cell wall components is important to support studies of both plant cell biology and also in relation to the industrial uses of cell wall materials. Cell walls are classed as either primary cell walls that prevent cell bursting and regulate cell expansion and or secondary cell walls, restricted to certain cell types, which have mechanical properties that resist external forces that would lead to cell collapse. Both types of cell wall are structurally complex composites with much of this complexity residing in sets of polysaccharides.

In most primary cell walls a load bearing network of cellulose microfibrils is crossed-linked with hemicelluloses and embedded in a matrix of pectic polysaccharides. Secondary cell walls are usually reinforced with the phenylpropanoid polymer lignin, but contain low amounts of pectins. Hemicellulosic polysaccharides include xyloglucan, xylan, mannan and glucan hemicelluloses and the pectic polymers include galacturonans, rhamnogalacturonans, arabinans, galactans and arabinogalactans (Albersheim *et al.*, 2010). Several of these polysaccharides can be varied in fine structural detail and several can be covalently interlinked within polymer networks.

There is considerable evidence that cell wall polysaccharide structures show taxonomic variation although our understanding of how polymer structure is integrated with both cell wall properties and plant evolution is limited (Bacic *et al.*, 1988; O'Neill & York, 2003; Popper & Fry, 2003, 2004; Matsunaga *et al.*, 2004; Popper *et al.*, 2004; Harris, 2005; Popper, 2008; Popper & Tuohy, 2010; Sørensen *et al.*, 2010; Popper *et al.*, 2011). While cell wall diversity is documented across the extant land plants, little is known of how the range of polysaccharides found in primary and secondary cell walls relate, if at all, to the evolution of specific cell wall functions and cell types during land plant evolution. A current view would suggest that there has not been a large innovation of new wall polymers but structural diversification of the same dozen or so polymer types and this may have involved modulation of specific functions for polysaccharide motifs throughout land plant evolution (Sørensen *et al.* 2010).

Our understanding of cell walls of early-diverging land plants (liverworts, mosses, hornworts, lycophytes and pteridophytes) is limited (Timell, 1962a,b; Bremner & Wilkie, 1971; Geddes & Wilkie, 1971, 1972; Basile *et al.*, 1989; Matoh *et al.*, 1996; Shimokawa *et al.*, 1999; Ligrone *et al.*, 2002; Popper & Fry, 2003, 2004; Matsunaga *et al.*, 2004; Peña *et al.*, 2008; Popper *et al.*, 2004; Fu *et al.*, 2004; Carafa *et al.*, 2005; Harris, 2005; Popper, 2006; Gómez Ros *et al.*, 2007; Leroux *et al.*, 2007; Fry *et al.*, 2008; Sørensen *et al.*,

2008; Martone *et al.*, 2009; Verhertbruggen *et al.*, 2009b; Marcus *et al.*, 2010). While these studies clearly indicate that the same major groups of polysaccharides are present in most cases, there is no clear view of how they conform to models of cell wall structure that have been developed for model and crop species such as *Arabidopsis* and maize. Indeed, a recent analysis of the early diverging fern *Equisetum* (Sørensen *et al.*, 2008; Fry *et al.*, 2008) has indicated structurally distinct cell walls that do not fit within either the type I or type II classification that had been developed for cell walls (Carpita & Gibeaut, 1993; Carpita, 1996). Although broadly useful, such a classification both neglected variation in polysaccharide content between cell types within organs and most notably did not relate to all plant species. Large-scale surveying of the structural and molecular diversity of cell walls is likely to provide fundamental insights into cell wall diversity in the contexts of both evolution and biology.

It is now commonly agreed that within the tracheophyte tree of life, a deep phylogenetic dichotomy occurred in the mid-Devonian, separating a group including the extant lycophytes from a group containing all ferns and seed plants, the euphyllophytes (Pryer *et al.*, 2004). The extant lycophytes comprise three main clades: homosporous clubmosses (e.g., *Lycopodium*), heterosporous quillworts (e.g., *Isoetes*) and spikemosses (e.g., *Selaginella*). The majority of extant ferns belong to the monophyletic leptosporangiate group which has diverged in an environment dominated by angiosperms (Schneider *et al.*, 2004), and therefore have faced selective pressures similar to those experienced by seed plants (Thomas & Spicer, 1987; Stewart & Rothwell, 1993). The remaining fern taxa belong to four eusporangiate lineages *sensu lato* (ophioglossoid ferns, marattioid ferns, horsetails and whisk ferns) which are the earliest diverged taxa among ferns (Supplemental Fig. 1).

Methods used to isolate and characterize cell wall polysaccharides are generally disruptive and lead to loss of all contextual information whether this relates to position within a cell wall or differences between cell types within an organ. Immunocytochemistry (IC) is an excellent method to compare cell walls within organs and also among taxa belonging to different plant groups. Sets of monoclonal antibodies (mAbs) have been developed that are directed to specific structural features of plant cell wall polysaccharides (Knox, 2008; Pattathill *et al.*, 2010). In addition to immunocytochemical analysis, we have also utilized these mAbs to probe microarrays of cell wall polysaccharides extracted from diverse fern species. This high throughput technique, known as Comprehensive Microarray Polymer Profiling or CoMPP, rapidly provides information about the relative abundance of epitopes present in cell wall material sequentially extracted with 1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid

and sodium hydroxide that semi-selectively release pectins and hemicelluloses from cell walls (Moller *et al.*, 2007; Sørensen *et al.*, 2009). Thus CoMPP also provides some insight into architectural features of cell walls and well as composition.

Here we report the use of CoMPP, to assess the occurrence of over 30 cell wall polysaccharide epitopes in 76 fern species and some related outliers such as the clubmoss *Lycopodium*. In order to study intrageneric variation we included 36 representatives of *Asplenium* and *Hymenasplenium*, the only genera within the Aspleniaceae. The aim of this study is to investigate the potential tissue-specific and taxonomically significant distribution of cell wall epitopes

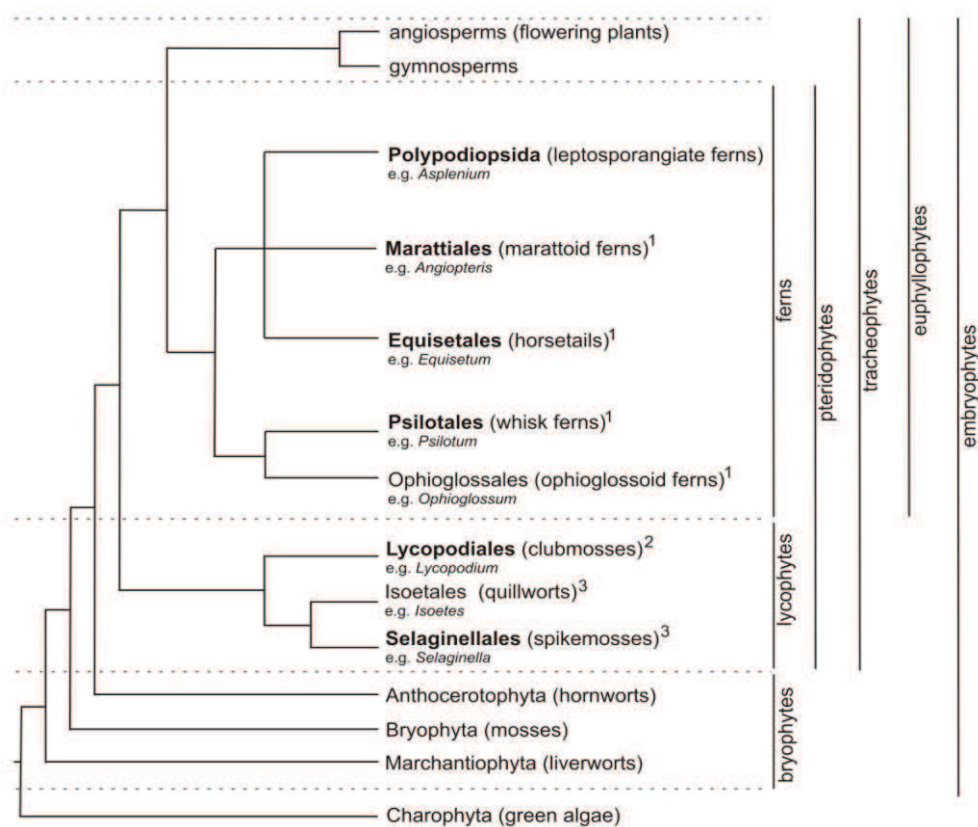


Figure 7.1: Schematic tree showing the relationships among some major land plant groups. 1: eusporangiate ferns s.l.; 2: homosporous lycophytes; 3: heterosporous lycophytes. Representatives of the plant groups indicated in bold were sampled for this study. Adapted from Smith *et al.*, 2006 and Qiu *et al.*, 2007.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

Seventy six fern species from 20 families with associated lycophytes (4 species) growing in the fern collection at the Gent University were sampled for material that was used in CoMPP assays with 34 cell wall directed molecular probes. A larger amount of samples of species belonging to the family Aspleniaceae (36 species) were collected to explore infrageneric variation. Species used in this study are listed in Appendix 1.

7.2.2 Comprehensive microarray polymer profiling (CoMMP)

CoMPP was performed as described elsewhere (Moller *et al.*, 2007). Fern petiole base samples were collected and processed into alcohol insoluble residues (AIR; see below). 5 g of AIR was extracted with 50mM CDTA (1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid), pH 7.5 and 4 M NaOH with 0.1% v/v NaBH₄. Printing of microarrays was performed using a Sprint microarrayer (ArrayJet, Roslin, Scotland, UK). Monoclonal antibody and carbohydrate-binding module specificity used in the CoMPP and immunolabelling studies are listed in Table 7.1 along with references. Secondary alkaline phosphatase-conjugated mAbs (anti-mouse, rat or his) were obtained from Sigma. Mean spot signals from three independent experiments are presented as a heatmap (created using the online tool available at http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi) with the values normalized to the highest value (set to equal 100). A cut off of 5% of the highest mean signal value was imposed and values below this are represented as 0.

7.2.3 Alcohol Insoluble Residue (AIR)

Material was suspended in liquid nitrogen and homogenized to a fine powder using a mortar and pestle. Five volumes of aqueous 70% ethanol were added and the suspension samples kept for 1 h at 4°C on a rotator. The suspensions were then centrifuged and the supernatant discarded. This procedure was repeated 5 times, before a final wash with acetone for 2 minutes. The AIR was then air dried.

7.2.4 Indirect immunofluorescence analysis of cell wall epitopes

Materials were fixed in 4% paraformaldehyde in 50 mM PIPES, 5 mM MgSO₄, and 5 mM EGTA. After washing in PBS, transverse sections were cut using a Microm HM650V vibration microtome (Thermo Fisher Scientific, Walldorf, Germany). Immunolabelling and section pretreatment with pectate lyase was carried out as described previously (Marcus *et al.*, 2008). A mixed-linkage glucan specific antibody (Biosupplies Australia, Victoria, Australia) was used as described by Meikle *et al.* (1994). Cellulose was stained with Calcofluor White M2R fluorochrome (fluorescent brightener 28; Sigma; 0.25 µg mL⁻¹ in dH₂O). Subsequently, all sections were washed in PBS buffer three times before mounting in a glycerol-based anti-fade solution (Citifluor AF1, Citifluor Ltd., UK).

Immunofluorescence was observed with a microscope equipped with epifluorescence irradiation (Olympus BX-61) and images were captured with a Hamamatsu ORCA285 camera and prepared with Volocity software.

7.2.5 Tissue printing on nitrocellulose

Cut surfaces of petioles were pressed firmly onto a nitrocellulose sheet. The prints were air dried for 30 min and immunolabeling was performed as described elsewhere (Willats *et al.* 1998).

Table 7.1: List of monoclonal antibodies used in this study

	mAb	Specificity	References
Pectic HG/related	2F4	unesterified, calcium cross-linked HG	Liners <i>et al.</i> , 1989, 1992
	JIM5	partially or un-esterified HG	Clausen <i>et al.</i> , 2003
	JIM7	partially methyl-esterified HG	Clausen <i>et al.</i> , 2003
	LM18	partially or un-esterified HG	Verhertbruggen <i>et al.</i> , 2009a
	LM19	partially or un-esterified HG	Verhertbruggen <i>et al.</i> , 2009a
	LM20	methyl-esterified HG	Verhertbruggen <i>et al.</i> , 2009a
	LM7	non-blockwise partially methyl-esterified HG	Willats <i>et al.</i> , 2001; Clausen <i>et al.</i> , 2003
RGI/related	LM5	(1→4)-β-galactan	Jones <i>et al.</i> , 1997
	LM6	(1→5)-α-arabinan	Willats <i>et al.</i> , 1998
	LM13		Verhertbruggen <i>et al.</i> , 2009b
	LM16		Verhertbruggen <i>et al.</i> , 2009b
	LM8	xylogalacturonan	Willats <i>et al.</i> , 2004
	LM9	feruloylated (1→4)-β-D-galactan	Clausen <i>et al.</i> , 2004
Hemicelluloses/XG	CCRC-M1	fucosylated xyloglucan	Puhlmann <i>et al.</i> , 1994
Hemicelluloses/xylan	LM15	XXXG-motif of xyloglucan	Marcus <i>et al.</i> , 2008
	LM10	(1→4)-β-D-xylan	McCartney <i>et al.</i> , 2005
	LM11	(1→4)-β-D-xylan / arabinoxylan	McCartney <i>et al.</i> , 2005
Hemicelluloses/mannan	LM21	mannan	Marcus <i>et al.</i> , 2010
Hemicelluloses/glucan	Callose	(1→3)-β-glucan	Meikle <i>et al.</i> , 1991
	MLG	(1→3,1→4)-β-glucan	Meikle <i>et al.</i> , 1994
AGPs	LM2	β-linked glucuronic acid	Smallwood <i>et al.</i> , 1996; Yates <i>et al.</i> , 1996
	LM14	AGP glycan	Moller <i>et al.</i> , 2008
	JIM4	AGP glycan	Knox <i>et al.</i> , 1989; Yates <i>et al.</i> , 1996
	JIM8	AGP glycan	Pennell <i>et al.</i> , 1991, 1992
	JIM13	AGP glycan	Knox <i>et al.</i> , 1991; Yates <i>et al.</i> , 1996
	JIM14	AGP glycan	Knox <i>et al.</i> , 1991; Yates <i>et al.</i> , 1996
	JIM15	AGP glycan	Knox <i>et al.</i> , 1991; Yates <i>et al.</i> , 1996
	JIM16	AGP glycan	Knox <i>et al.</i> , 1991; Yates <i>et al.</i> , 1996
	MAC207	AGP glycan	Pennell <i>et al.</i> , 1989
	JIM11	Extensin	Smallwood <i>et al.</i> , 1994
Extensins	JIM12	Extensin	Smallwood <i>et al.</i> , 1994
	JIM19	Extensin	Smallwood <i>et al.</i> , 1994
	JIM20	Extensin	Smallwood <i>et al.</i> , 1994
	LM1	Extensin	Smallwood <i>et al.</i> , 1995

HG= homogalacturonan, RG-I = rhamnogalacturonan-I, XG = xyloglucan, MLG = mixed-linkage glucan, AGP = arabinogalactan-protein.

7.3 RESULTS

Interpretation of CoMPP data was approached from the perspective of cell wall polysaccharide classes and the results are presented as heatmaps (Figs. XX) in which mean spot signals from the CoMPP arrays are correlated to colour intensity, and the highest value in the entire data set is set to 100 and all other values adjusted accordingly. After an initial CoMPP analysis of roots, rhizomes, petiole base, transition petiole-rachis, rachis top, as well as lamina of *Asplenium elliotii* (Fig. 1), we focused on the comparative analysis across species of petiole bases as a) the petiole base exhibits the highest diversity in tissues and tissue types, b) we found the highest number of distinct epitopes in the petiole base and c) the stiffness of fern petioles enabled efficient sectioning and tissue printing. These large data sets are shown in relation to both fern division and molecular probe class in Supplemental Figures 2 and 3. These analyses resulted in a very large amount of information indicating the varied occurrence of certain epitopes and in several cases findings were explored further with supportive immunochemistry (IC) and tissue printing (TP). Broad themes that became apparent in the CoMPP analysis included the observation that the majority of the epitopes characterized in angiosperms were widely present across the assessed fern species. Probes for mixed-linkage glucans, and callose were utilised in the analysis and the corresponding epitopes were widely detected, especially in the NaOH fractions. Extensin epitopes (particularly the JIM11 and LM1 epitopes) displayed scattered diverse patterns of detection across the ferns and a lack of consistent patterns was observed by IC. These cell wall components were not pursued in the current work where the focus of the supporting IC and TP analysis was on sets of pectic, hemicellulosic and arabinogalactan-protein (AGP) epitopes. Though we occasionally observed incongruence between and/or variation within our CoMPP- and IC results, our broad sampling within the ferns, and within the Aspleniaceae in particular, allowed the clear documentation of patterns of epitope occurrence. In several cases no binding of specific antibodies above background was detected in the CoMPP or in the IC analyses which indicated that the epitopes were absent or of low-abundance.

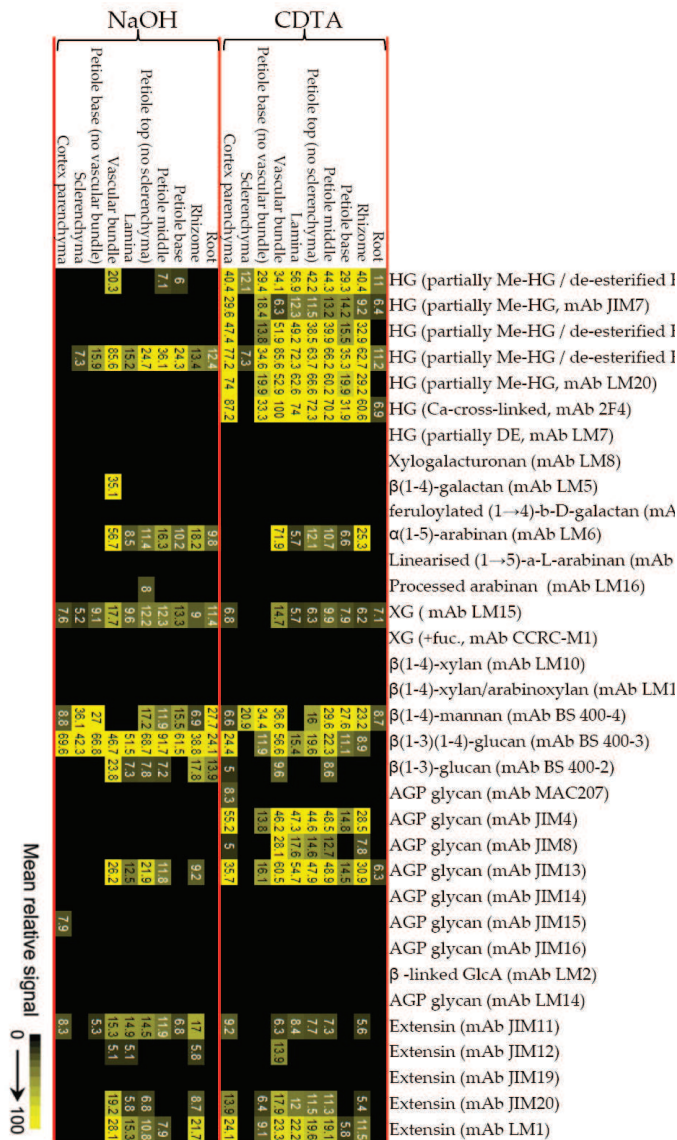


Figure 7.2:
Comprehensive
microarray polymer
profiling (ComPP)
heatmap of CDTA
and NaOH
extractions of total
organ or isolated
tissue(s) of
Asplenium elliptii.
The probes are listed
at the top of the
heatmap. References
for probe specificity
are listed in table 7.1.
Abbreviations: HG:
homogalacturonan;
AGP:
arabinogalactan
protein.

Figure 7.3 and 7.4: Comprehensive microarray Polymer Profiling (ComPP) heatmap of CDTA (Fig. 7.3) and NaOH (Fig. 7.4) extractions of 76 fern and 4 lycophyte species.

Plant taxa are listed at the left and probes on top of the heatmaps. References for probe specificity are listed in table 1. Abbreviations: HG: homogalacturonan; AGP: arabinogalactan protein. Abbreviations: ly: lycophytes; h: horsetails; m: marattoid ferns; w: whisk ferns; le: leptosporangiate ferns.

Figure 7.3

Figure 7.4

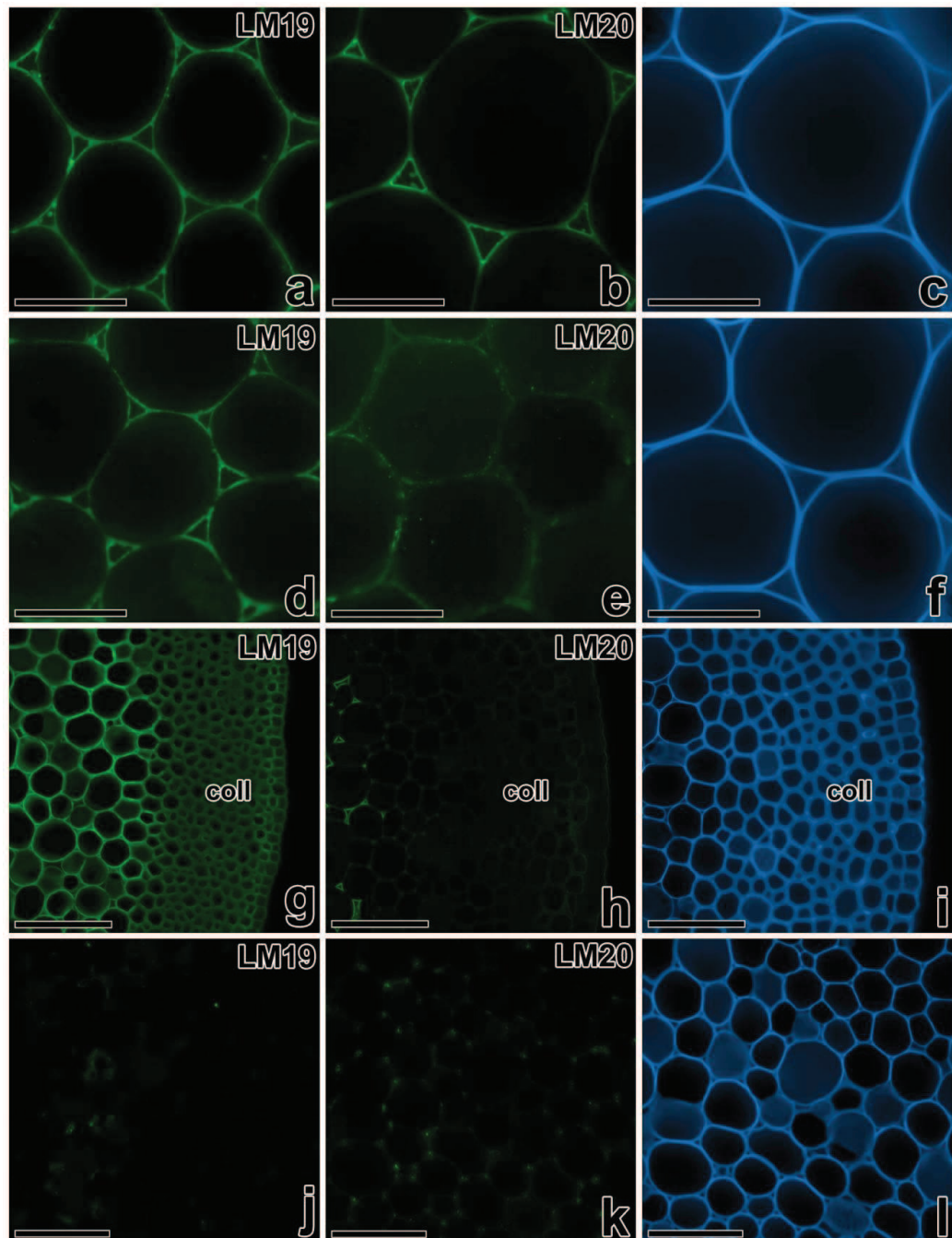
Figure 7.4

Pectic homogalacturonan (HG) epitopes

Homogalacturonan (HG) is the major pectic polysaccharide and a range of monoclonal antibody probes are available that recognize subtly different methyl- esterification patterns of this polymer (Willats *et al.*, 2001; Clausen *et al.*, 2003; Verhertbruggen *et al.*, 2009a).

In the CoMPP analysis pectic HG was widely detected in the CDTA extracts by JIM5, JIM7, LM18, LM19, LM20 and 2F4 antibodies as expected (Fig. 7.2, 7.3, 7.4) and the presence of HG epitopes and differential occurrence was confirmed by IC (Fig. 7.5). A distinctive feature of the IC was that the LM19 epitope (unesterified HG) was generally more abundant relative to the LM20 epitope (methylesterified HG). LM19 bound to primary cell walls, whereas LM20 had a more restricted binding pattern to the middle lamellae and intercellular space linings (Fig. 7.5a-f) – the converse to what has been observed in angiosperm parenchyma systems. The prevalence of the LM19 epitope over the LM20 epitope was also apparent in collenchymatous cell walls (Fig. 7.5g-i). In the case of the whisk fern *Psilotum* and also the lycophytes studied only low levels of pectic HG epitopes were detected in the CDTA extracts. IC confirmed these results as shown for *Lycopodium* in Figure 7.5j,k. The LM7 pectic HG epitope associated with cell walls at intercellular spaces and the LM8 xylogalacturonan epitope associated with cell detachment in angiosperms were not detected by CoMPP or IC/TP analyses in any of the species studied. Cortical parenchyma cell walls in some species (e.g., *Asplenium trichomanes*, *Davallia sp.*) displayed significant autofluorescence and IC showed no binding of any of the pectic HG antibodies in these cases.

Figure 7.5: Indirect immunofluorescence detection of homogalacturonan epitopes with low (LM19) and high (LM20) levels of esterification. Calcofluor White fluorescence (c, f, i, l) shows the full extent of cell walls. (a-c) Transverse sections of a *Asplenium daucifolium* petiole showing binding of LM20 to the middle lamellae and intercellular space corners, while LM19 binds to primary cell walls. (d-f) Transverse section of a *Woodwardia radicans* petiole showing that the LM19 antibody binds abundantly compared to the LM20 antibody. (g-h) The prevalence of the LM19 epitope over the LM20 epitope is also apparent in transverse sections of *Asplenium theciferum* petioles, where LM19 binds abundantly to collenchymatous cell walls. (j-l) LM19 and LM20 weakly bind to primary cell walls in a transverse section of a *Lycopodium squarrosum* stem. Abbreviations: coll: collenchymatous tissue. Scalebars: 40 µm.



1,5-arabinan and 1,4 galactan epitopes

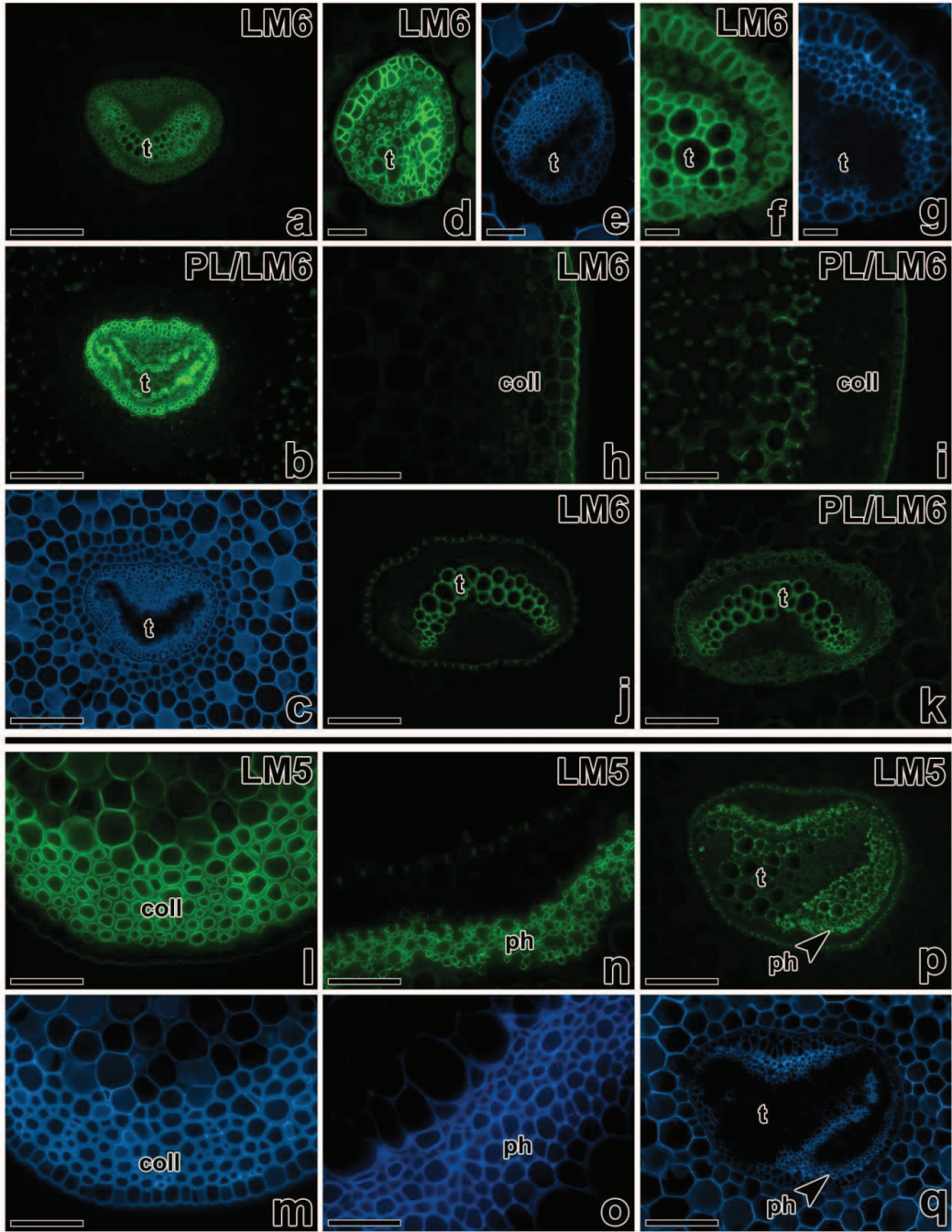
Analysis of the pectic component RG-I was performed by means of the arabinan and galactan-directed monoclonal antibodies, LM6 and LM5 respectively, and these epitopes emerged from the CoMPP and IC analyses with distinct distribution patterns with little overlap. 1,5-arabinan and related polymers are present in the complex heterogeneous pectic polymer RG-I (Caffell & Mohnen, 2009), but can also be present in arabinogalactan proteins (Lee *et al.*, 2005).

In the CoMPP-analysis the arabinan LM6-epitope was detected in the CDTA and NaOH extracts of most species, with relative high amounts in *Equisetum* (horsetails) and marattoid ferns, and absent in homosporous lycophytes (Fig. 7.2, 7.3, 7.4). Two arabinan-related epitopes, LM13 and LM16, which appear to reflect aspects of arabinan processing (Verhertbruggen *et al.*, 2009b) were only detected in *Equisetum* and *A. daucifolium* respectively. In isolated vascular bundles of *A. elliotii* (Fig. 7.2) the LM6 epitope was relatively highly abundant. Supportive IC and TP showed that the distribution of the LM6-epitope was consistent among the leptosporangiate ferns (Fig. 7.6, 7.10). LM6 bound specifically and strongly to the parenchymatous cell types of the vascular bundle - the phloem parenchyma and the pericycle (Fig. 7.6a-g), as well as to epidermal cell walls (Fig. 7.6h,i). Within the Aspleniaceae, LM6-binding seemed to be restricted to *Asplenium* species, but after pectin unmasking it was detected in *Hymenasplenium* (Fig. 7.6j,k). Pectate lyase treatment also unmasked LM6-epitopes in the cortex parenchyma cell walls in *Asplenium* (Fig. 7.6h,i). In the eusporangiate ferns (*Equisetum*, *Angiopteris* and *Psilotum*) LM6 strongly bound to all cortical cell walls confirming the relative high amounts of the LM6 epitope detected in these species in the CoMPP-analysis. In *Lycopodium* we observed a very weak LM6-binding. Generally, the LM13-epitope showed similar distribution patterns as the LM6-epitope, but binding was very weak. LM16 was not found to bind to any fern cell walls, except for *Equisetum* where a restricted occurrence of this epitope was observed in subsidiary cells.

In the CoMPP-analysis, the galactan LM5 epitope was detected in the NaOH extract of most leptosporangiate ferns and lycophytes (Fig. 7.2, 7.3, 7.4). In a sample with isolated vascular bundles of *Asplenium elliotii* (Fig. 7.2) a high abundance of LM5 was detected in the vascular bundles. In *Equisetum* and *Danaea*, high relative amounts were detected in both the NaOH and CDTA extractions. The LM9 feruloylated galactan epitope was not detected in any of the species studied. IC showed the presence of LM5 in the inner cell wall layers of collenchymatous tissues (e.g., *Asplenium theciferum*, *Asplenium loxoscaphoides*, *Asplenium compressum*, *Asplenium tenerum*) as shown for *A. theciferum* in

Figure 7.6l,m. LM5 also bound to cortical parenchyma of most of these ferns (Fig. 7.6l), but weakly. In addition, the LM5 epitope was detected in sieve cells of all leptosporangiate ferns as showed for *Asplenium* and *Blechnum* in Figure 3n-q. In *Equisetum*, LM5 strongly bound to the inner cell wall layers of collenchymatous tissues as well as to the cortical parenchyma. In *Lycopodium*, a strong binding to cortical parenchyma and a weaker binding to phloem cells was observed. Pectin lyase treatment generally resulted in stronger binding of LM5, but did not unmask the epitope in tissues where no LM5 epitope was detected before the treatment.

Figure 7.6: Indirect immunofluorescence detection of arabinan (LM6) and galactan (LM5) epitopes. Calcofluor White fluorescence (e, g, c, m, o, q) shows the full extent of cell walls. **(a-c)** Transverse section of *Asplenium theciferum* petioles labelled with LM6 with (b) and without (a) pectate lyase pre-treatment (PL) showing the abundance of LM6-epitopes in the parenchymatous cell walls of the vascular bundle. **(d-g)** Similar distribution patterns of the LM6-epitope are found in transverse sections of *Todea sp.* (d-e) and *Blechnum brasiliense* (f-g) petioles. **(h-i)** LM6 also binds to epidermal cell walls of *A. theciferum* petioles and pectate lyase pre-treatment (PL) unmasked the LM6-epitope in the cortical parenchyma (i). **(j-k)** LM6 is only detected in *Hymenasplenium obscurum* vascular bundles after enzymatic removal of pectic HG (k). **(l-m)** Binding of LM5 to the innermost cell wall layers of collenchymatous tissue in transverse sections of *Asplenium theciferum*. **(n-o)** Abundance of the LM5 epitope in cell walls of phloem sieve cells of *Asplenium compressum*. **(p-q)** Similar distribution pattern of LM5 in a transverse section of a *Blechnum brasiliense* petiole. Abbreviations: coll: collenchymatous tissue; ph: phloem. Note the autofluorescence of tracheid cell walls (t). Scalebars: a, b, c, p, q: 200 µm; d, e, f, g, h, i, j, k, l, m, n, o: 100 µm.



Xyloglucan epitopes

Xyloglucans have a backbone of (1→4)- β -D-glucan and some glucosyl residues are substituted with short side chains (Albersheim *et al.*, 2010). Both LM15, binding to the XXXG-motif of xyloglucan, and CCRC-M1, binding to fucosylated xyloglucan were employed.

CoMPP-analysis indicated the presence of the LM15 epitope in the NaOH extractions of most of the species studied, including species of the Aspleniaceae and other leptosporangiate ferns, as well as eusporangiate ferns and lycophytes (Fig. 7.2, 7.3, 7.4). The CCRC-M1 epitope, on the other hand, was detected in the NaOH extraction of most leptosporangiate and eusporangiate ferns studied, but - with the exception of very weak presence in two species - not detected in the Aspleniaceae. Within the lycophytes, the CCRC-M1 epitope was only detected in the heterosporous *Selaginella*. IC showed weak binding of LM15 to phloem cell walls in most of the ferns studied (Fig. 7.7a,b). After pectate lyase treatment this binding was stronger and we also observed binding to cortical parenchyma (Fig. 7.7b,c,d). Binding of LM15 to phloem cell walls was also observed in *Equisetum* and *Lycopodium* (Fig. 7.7e,f). CCRC-M1 did not bind to any of the *Asplenium* and *Hymenasplenium* species, nor to any homosporous lycophytes studied (Fig. 7.7g,h), treated or untreated with pectate lyase. In non-asplenioid ferns, it bound to phloem cell walls (Fig. 7.7i,j). Though high relative amounts of the CCRC-M1 epitope were detected in our CoMPP-analysis, IC only revealed weak binding, even after pectate lyase treatment.

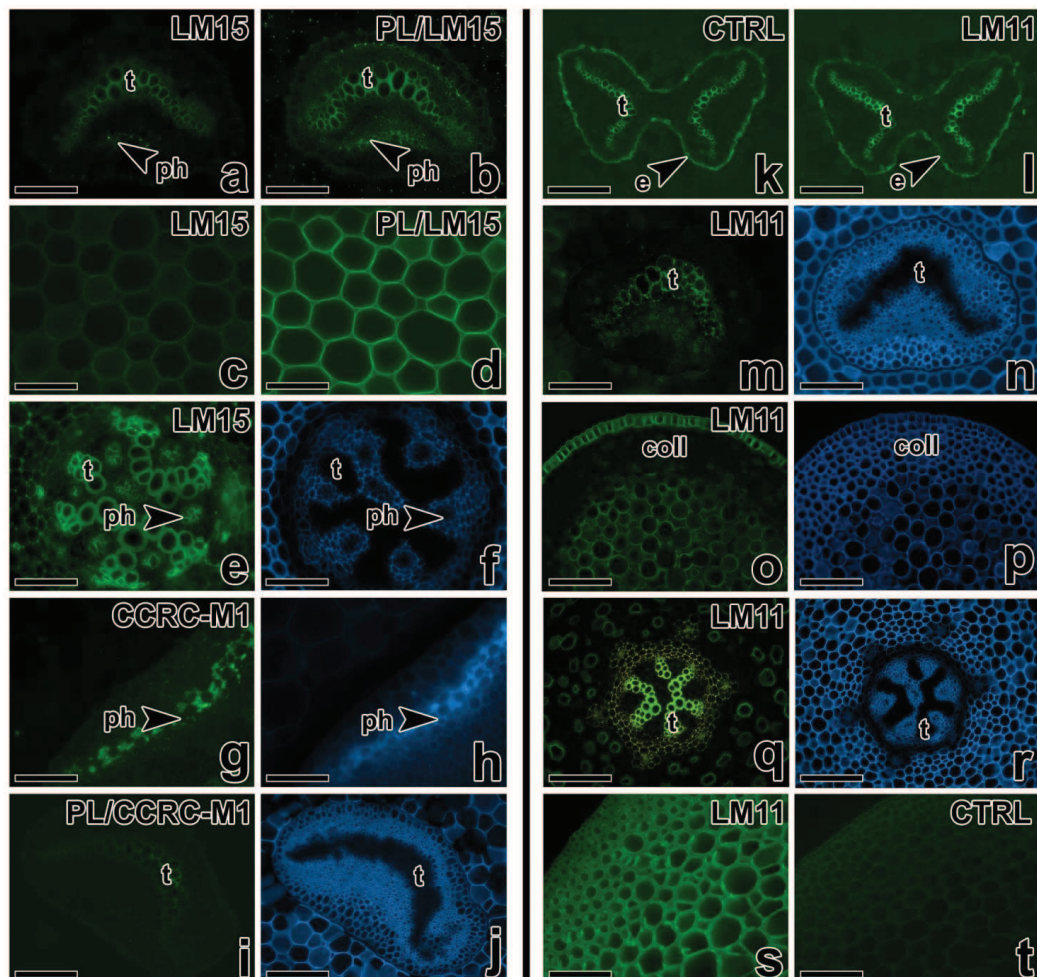
Xylan epitopes

The monoclonal antibodies LM10 and LM11 both recognise unsubstituted (1→4)- β -xylan, but LM11 can also bind to relatively highly substituted arabinoxylans (McCartney *et al.*, 2005).

CoMPP-analysis only detected the LM10 epitope in *Diplazium* and the LM11 epitope in the NaOH extraction of a number of leptosporangiate ferns and heterosporous lycophytes (Fig. 7.2, 7.3). LM10 and LM11 epitopes were not detected in Aspleniaceae, eusporangiate ferns and homosporous lycophytes. With few exceptions, LM11 gave much the same IC results, binding to tracheid cell walls (Fig. 7.7k,l) of ferns and lycophytes. However, in several cases autofluorescence obscured observation of binding patterns as shown for *A. polyodon* in Figure 7.7k,l. In a few cases LM11 bound to phloem parenchyma cells (Fig. 7.7m,n), and to cortical and epidermal primary cell walls - but not

to collenchymatous cell walls - as shown for *A. rutifolium* (Fig. 7.7o,p), but these labelling patterns were not found in closely related species. In the homosporous *Lycopodium squarrosus*, LM11 bound to scattered cells of the cortex parenchyma (Fig. 7.7q,r), whereas in the heterosporous lycophyte *Selaginella* it strongly bound to all cortical parenchyma cell walls (Fig. 7.7s,t). We observed no binding of LM10 to any fern or lycophyte species studied, but weak binding could have been obscured by autofluorescence of tracheid cell walls.

Figure 7.7: Indirect immunofluorescence detection of xyloglucan (LM15, CCRC-M1) and xylan (LM11) epitopes. Calcofluor White fluorescence (f, h, j, n, p, r) shows the full extent of cell walls. **(a-d)** Pectate lyase treatment (PL) unmasks LM15-epitopes in phloem cell walls of *Asplenium theciferum* petioles (b) and in primary cell walls of cortical parenchyma of *Asplenium elliotii* petioles (d). **(e-f)** Binding of LM15 to phloem cells in a transverse section of a *Lycopodium squarrosus* stem. **(g-h)** Binding of CCRC-M1 to phloem cell walls in vascular bundles of *Drynaria cf. mollis* petioles. **(i-j)** CCRC-M1 epitopes are not detected in *Asplenium currorii* petioles, even after pectate lyase pre-treatment (PL). **(k-l)** Autofluorescence of tracheids (t) obscures observation of LM11 binding (l) in transverse sections of *Asplenium polyodon* petioles. Note also the autofluorescence of the suberin lamellae of the endodermis (e) **(m-n)** LM11 binding to tracheid cell walls and phloem cell walls of *Asplenium loxoscaphoides*. **(o-p)** Abundance of LM11 epitopes in epidermal cell walls and cortical parenchyma of *Asplenium rutifolium*. **(q-r)** Binding of LM11 to cell walls of scattered cortical parenchyma cells in a transverse section of a *Lycopodium squarrosus* stem. **(s-t)** In *Selaginella grandis* stems, LM11 epitopes are detected in all cortical parenchyma cell walls. Abbreviations: coll: collenchymatous tissue; ph: phloem. Note the autofluorescence of tracheid cell walls (t). Scalebars: a, b, c, d, e, f, g, h, m, n, s, t: 100 µm; i, j, k, l, o, p, q, r: 200 µm.

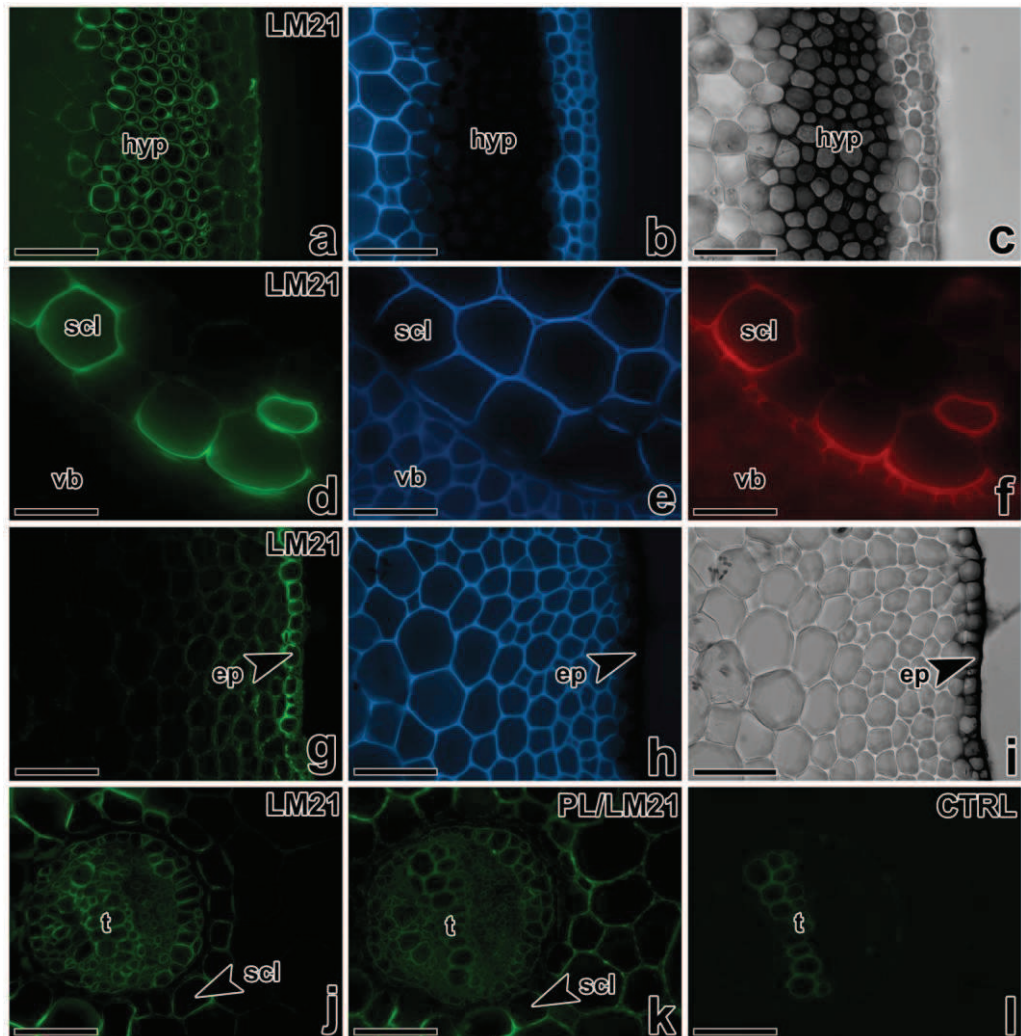


Mannan-epitopes

Mannans are a complex set of hemicellulosic heteroglycans that can contain backbone glucosyl and/or side branch galactosyl residues and are proposed to have storage and structural functions in cell walls (Scheller & Ulsskov, 2010).

CoMPP detected mannans in both NaOH and CDTA extractions of ferns and lycophytes (Fig. 7.2, 7.3, 7.4). High amounts of mannan-epitopes were detected in species with relative high amounts of sclerified tissues. In *A. elliotii* the highest amount of mannan-epitopes was detected in isolated hypodermal sclerenchyma, petiole base tissue and vascular bundles, whereas no mannan-epitopes were detected in the lamina (Fig. 7.2). IC showed that, throughout all ferns and lycophytes studied, the LM21-epitope is strongly associated with sclerified secondary cell walls. LM21 strongly bound to the inner cell wall layer of sclerified epidermal and hypodermal cell walls, scattered sclerified cortex parenchyma cells as well as to xylem tracheids and sclereids surrounding the vascular bundles (Fig. 7.8a-i). In some cases we observed weak binding of LM21 to cortical parenchyma and the vascular bundle. After pectate lyase treatment LM21 bound to many primary cell walls (Fig. 7.8j-o). Tissue prints revealed the partial solubility of mannan epitopes of secondary cell walls (Fig. 7.10).

Figure 7.8: Indirect immunofluorescence detection of mannan (LM21) epitopes. Calcofluor White fluorescence (b, e, h) and bright-field (c, i) shows the full extent of cell walls. **(a-c)** LM21 binding to the innermost cell wall layer of sclerenchyma hypodermal cells (hyp) and sclereids of a transverse section of an *Asplenium elliotii* petiole. **(d-f)** Binding of LM21 to the cell walls of sclereids (scl) surrounding the vascular bundle (vb) of *Asplenium elliotii*. Red autofluorescence of sclereid cell walls (f) indicates the presence of phenolic compounds. **(g-i)** Immunodetection of LM21 in sclerified epidermal cell walls of *Hymenasplenium obscurum*. **(j-l)** Unmasking of LM21 epitopes in primary cell walls of the cortical parenchyma of *Elaphoglossum sp.* petioles after pectate lyase pre-treatment (PL). Note that LM21 binding to vascular bundles in *Elaphoglossum* does not require pectate lyase pre-treatment. Abbreviations: scl: sclerenchyma; ph: phloem; ep: epidermis. Dark cell walls in the bright field images (c, i) indicate that these are impregnated with phenolic compounds. Note the autofluorescence of tracheid cell walls (t). Scalebars: a, b, c, g, h, i, j, k, l: 100 µm; d, e, f: 50 µm.



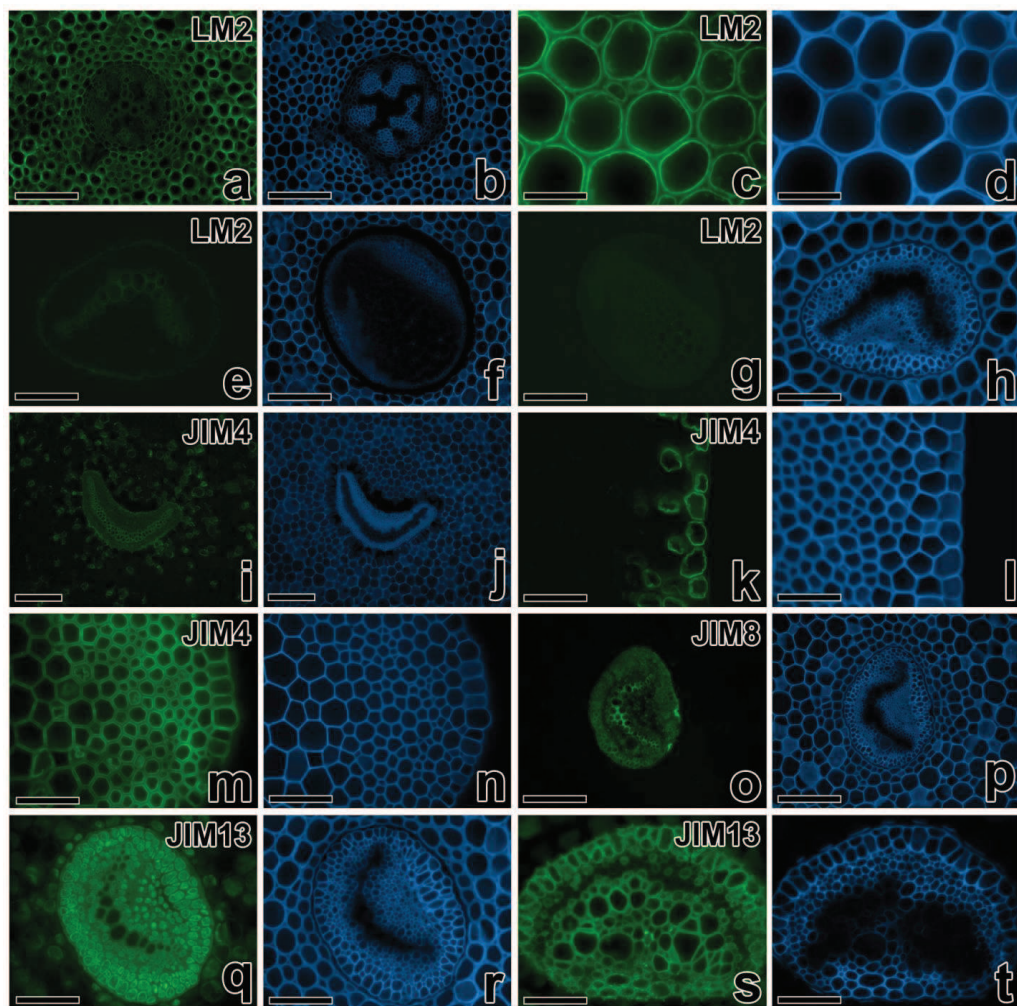
Glycan epitopes of arabinogalactan-proteins

AGPs are a family of highly glycosylated hydroxyproline-rich glycoproteins (HRGPs) and a series of antibodies are available that indicate the extensive developmental regulation of AGP glycan components in a range of plant systems, mainly in association with plasma membranes (Showalter, 2001).

In the CoMPP-analysis, we detected relatively high amounts of the LM2 epitope in the NaOH extractions of *Lycopodium* cell walls, but, except for two species where we detected a low abundance – it was absent in all other ferns studied (Fig. 7.3, 7.4). In contrast, high levels of the distinct JIM4 and JIM13 AGP-epitopes were found in the CDTA extractions from across the fern species where JIM8 and MAC207 were also detected but only weakly (Fig. 7.3, 7.4).

IC showed strong binding of LM2 to cell membranes of *Lycopodium* (Fig. 7.9a-d). It did not bind to any of the other ferns or heterosporous lycophytes studied (Fig. 7.9e-h). TP confirmed these results and showed strong binding of LM2 in *Lycopodium* cortex parenchyma (Fig. 7.10). JIM4 bound weakly to plasma membranes of the cortical parenchyma, epidermis and parenchymatous cell types of the vascular bundles (Fig. 7.9i-n). Similar binding patterns were obtained with JIM8 and JIM13 (Fig. 7.9o-t), with JIM8-binding being weaker in most cases compared to JIM13, especially in the cortical parenchyma cell walls. MAC207 also bound in a similar way as JIM4, but even weaker when compared to JIM8.

Figure 7.9: Indirect immunofluorescence detection of glycan epitopes of arabinogalactan-proteins (LM2, JIM4, JIM8, JIM13). Calcofluor White fluorescence (b, d, f, h, j, l, n, p, r, t) shows the full extent of cell walls. **(a-d)** Strong binding of LM2 to cell membranes of cortical parenchyma cells of *Lycopodium squarrosum* stems. **(e-h)** No binding of LM2 to any tissues of *Asplenium rutifolium* and *Drynaria cf. mollis* petioles. **(i-l)** Binding of JIM4 to cell membranes in the vascular bundle and cortical parenchyma (i) as well as in the epidermis (k). **(m-n)** Strong binding of JIM4 to cortical parenchyma and epidermal cell membranes in *Hymenasplenium obscurum* petioles. **(o-p)** Abundance of JIM8 epitopes in the vascular bundle of an *Asplenium loxoscaphoides* petiole. **(q-t)** Strong binding of JIM13 to cell membranes of parenchymatous cell types in the vascular bundles of *Asplenium theciferum* (q-r) and *Blechnum brasiliense* (s-t) petioles. Scalebars: a, b, g, h, k, l, o, p: 200 μm ; c, d: 50 μm ; e, f, m, n, q, r, s, t: 100 μm .



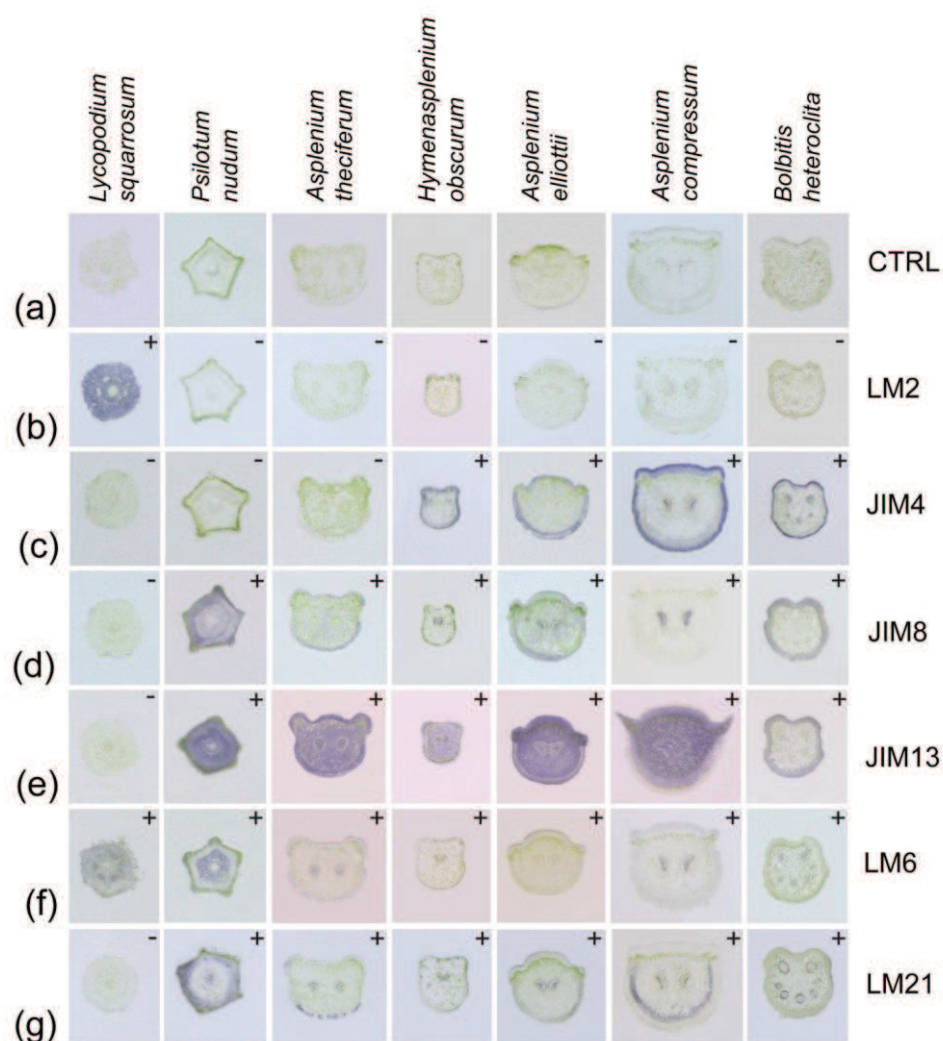


Figure 7.10: Tissue printing of one lycophyte (*Lycopodium squarrosum*) and six ferns (*Psilotum nudum*, *Asplenium theciferum*, *Hymenasplenium obscurum*, *Asplenium compressum*, and *Bolbitis heteroclita*). Unlabelled control tissue prints are shown in (a). Immunodetection of glycan epitopes of arabinogalactan proteins are shown in b-e. Immunolabelling with the anti-arabinan antibody LM6 and the anti-mannan antibody LM21 are shown in f and g respectively.

7.4 DISCUSSION

Even though most of the molecular probes used in this study have been raised against wall epitopes from more advanced plant taxa, they are clearly applicable to the analysis of fern cell walls. This study indicates also that many of the structures present in angiosperm cell wall polysaccharides are conserved. It is possible that other probes directed to fern-specific structural features of cell wall polysaccharides are required for a full analysis of fern cell walls. The major findings of this study are that some cell wall polysaccharide structures show taxonomically significant and/or tissue and tissue-type-specific distributions.

Reduced levels of pectic homogalacturonan (HG) in lycophytes and differential occurrence of pectic (HG) epitopes in ferns

Pectic polysaccharides are abundant components of the primary cell walls of most angiosperms, except in the grasses where the pectic content is low (Carpita, 1996) and have been found in green algae (Domozych *et al.*, 2007). Recent studies showed that pectic polysaccharides are also abundant in fern cell walls (Popper & Fry, 2004; Matsunaga *et al.*, 2004; Leroux *et al.*, 2007) and we found pectic HG in the primary cell walls of most the fern species studied. Relatively low levels of HG were found in all Lycophytes and *Psilotum* in our CoMPP-assay as well as in our labelling experiments, indicating that pectin is not a major constituent of their cell walls, which is supported by previous analyses where low levels of galacturonic acid were found (Popper & Fry, 2003). We obtained no evidence for the presence of LM7 HG epitope in fern cell walls in either CoMPP or immunolabelling. This epitope which is a specific methyl-esterification pattern of HG has been reported to be present in cell walls of angiosperms (Willats *et al.*, 2001) and algae (Domozych *et al.*, 2007). It is of interest that the probe for unesterified HG, LM19, bound throughout fern cell walls more widely than LM20, which is directed to a methyl-esterified epitope. The LM20 epitope was most detected at the lining of intercellular spaces. This is the converse of that generally seen in angiosperm parenchyma tissues and may indicate that methyl-esterification of HG, and its *in muro* de-esterification, has a distinct functional status in this group of plants.

1,5-arabinan and 1,4 galactan epitopes associated with, but not always restricted to, specific tissues and/or cell types

RG-I is a highly variable set of polysaccharides both in its structure and occurrence within cell walls (Willats *et al.*, 1998, 1999; Bush and McCann, 1999; Verhertbruggen *et al.*, 2009b). Many studies have suggested that the structures of RG-I side chains can vary

during development (Skjot *et al.*, 2002; Lee *et al.*, 2005). In angiosperms with complex stratified meristems LM5 and LM6 epitopes have been implicated in meristem functions (Willats *et al.*, 1999; Serpe *et al.*, 2001; McCartney *et al.* 2003) and both have been immunodetected in mature tissues of algae (Domozych *et al.*, 2010), gymnosperms (Arend, 2008), and angiosperms (Jones *et al.*, 1997). A remarkable fact is the detection of high amounts of LM5 in the CDTA cell wall extractions for all *Equisetum*-species and *Danaea*, showing that they have a distinct cell wall architecture compared to all other ferns and lycophytes studied where the LM5 epitope required NaOH for extraction. Additionally, the amounts of LM6 epitopes in *Equisetum* and *Danaea* were also among the highest obtained in both the CDTA and NaOH cell wall extractions. Immunocytochemistry further supported these observations as LM6, which, in contrast to the leptosporangiate ferns - where it was largely restricted to the vascular bundle and epidermis - was immunodetected in all tissues in *Equisetum* and Marattoid ferns. These findings are in accordance with some recently proposed phylogenetic studies presenting evidence that Marratiaceae and Equisetaceae are closely related (Pryer *et al.*, 2004). Within the leptosporangiate Aspleniaceae family, we showed that LM6 and LM13 arabinan probe binding was taxonomically restricted to *Asplenium* species. However, these epitopes were detected in both *Asplenium* and *Hymenasplenium*-species in our CoMPP-analysis and after pectic lyase treatment of sections indicating a taxonomic pattern of epitope masking that may reflect low abundance. The distribution of the LM5 and LM6 epitopes, being restricted to specific tissues and/or cell types – the equivalent of which has not been reported before, was consistent throughout the leptosporangiate ferns. It has been postulated that the occurrence of RG-I and its structural variants can be related to mechanical properties of cells or developing organs (McCartney *et al.*, 2000; Jones *et al.*, 2003; Ulvskov, 2005). Notwithstanding the fact that structure-function relationships of galactan-rich pectins are still poorly understood, the literature (Willats *et al.*, 1999; Bush *et al.* 2001; McCartney *et al.*, 2003) suggests that these polymers might play an important structural and/or regulatory role in mechanically stressed cell walls. Correspondingly, we immunodetected LM5-epitopes in the walls of sieve cells and collenchymatous cells, two cell types which undergo extensive elongation during differentiation.

Xyloglucan epitopes show clear taxonomical restrictions or are associated with phloem tissues and, after unmasking, primary cell walls.

Xyloglucans are the most abundant hemicelluloses in primary walls of seed plants, except for grasses (Scheller & Ulvskov, 2010; Popper & Fry, 2004), where (glucurono)arabinoxylans are the major hemicelluloses (Carpita & Gibeaut, 1993). They

have been detected in primary cell walls of bryophytes (Popper & Fry, 2003, 2004; Kremer *et al.*, 2004, Ligrone *et al.*, 2002), lycopodiophytes, ferns and gymnosperms (Popper & Fry, 2004) and immunolabelling experiments showed the presence of xyloglucan-epitopes in some charophycean green algae (Domozych *et al.*, 2009a,b). Both the CoMPP-analysis and IC revealed that the occurrence of CCRC-M1, directed against fucosylated xyloglucan, showed clear taxonomical restrictions, being absent in Aspleniaceae and homosporous lycophytes. In contrast, the LM15 monoclonal antibody, recognising the XXXG-motif of xyloglucan did not display any taxonomically significant distribution patterns. This epitope was associated - but not always restricted - to the phloem of all ferns and lycophytes studied, suggesting that xyloglucan might play an important structural or functional role in phloem cell walls of tracheophytes. Pectate lyase treatment unmasked LM15 epitopes in primary cell walls confirming that fern primary cell walls indeed contain xyloglucans.

Xylan epitopes are associated with secondary cell walls but also display some specific distribution patterns

Xylans are the major cellulose-linking polysaccharides in secondary cell walls of higher plants (Harris, 2005; Scheller & Ulsvkov, 2010), but they have also been found in charophycean green algae (Domozych *et al.*, 2009a), chlorophytes, and red algae (Lahaye *et al.*, 2003). In commelinid monocots, xylans are the major non-cellulosic polysaccharides in primary cell walls (Scheller & Ulsvkov, 2010). In ferns, xylans have been isolated from secondary cell walls of *Osmunda cinnamomea* (Timmel, 1962a). In a comparative study on tracheophytes Carafa *et al.* (2005) showed that LM10 and LM11 gave much the same labelling patterns with few exceptions, labelling xylem tracheids and vessels and, where present, sclerenchyma. As expected, LM11 bound to the tracheid cell walls in all the ferns and lycophytes studied. In contrast with the IC results, CoMPP-results, no signals above background were detected in most of the tissues in both the EDTA and NaOH cell wall extractions. This could be explained by the fact that, in most ferns, tracheids only comprise a small fraction of the total amount of tissue. The highest amount of xylan epitopes was found in *Cyathea*, a tree fern, which, in this study, was the sample with the highest xylem to total tissue ratio. Moreover, only low levels of xylan-epitopes have been detected in isolated vascular bundles and sclerenchyma of *A. elliotii* suggesting that xylans might not be the major hemicellulosic compounds in all fern secondary cell walls. The unusual binding pattern of LM11 in *Lycopodium squarrosum* stems, restricted to scattered parenchyma cell walls, suggests that differentiation or modification of cortical parenchyma might not occur simultaneously in all parenchyma cells, and that

immunolabelling of older stems could show the presence of these epitopes in all cell walls.

Mannan-epitopes are associated with sclerified secondary cell walls and unveiled in primary cell walls after pectate lyase treatment.

Mannans appear to be very abundant in the primary cell walls of the earliest land plants such as bryophytes, lycophytes, and early-diverging ferns, and less abundant in leptosporangiate ferns, gymnosperms and angiosperms (Popper and Fry, 2003, 2004; Nothnagel & Nothnagel, 2007). In primary cell walls of fern and lycophyte taxa such as *Lycopodium*, *Psilotum* and *Equisetum*, high concentrations of mannose-containing polysaccharides have been found (Bailey & Pain, 1971; Popper & Fry, 2004). They have also been detected in algal species, some of which completely lack cellulose in their cell walls (Frei and Preston, 1968; Preston, 1968). In angiosperms, glucomannans, galactoglucomannans, and galactomannans are usually found at low levels in secondary cell walls (Bacic *et al.*, 1988; Carpita & McCann, 2002; Harris, 2005), whereas they are the major hemicelluloses in secondary cell walls of many gymnosperms (Bacic *et al.*, 1988; Harris, 2005). Our results confirm these observations. Particularly, the LM21 epitope is associated with sclerified or sclerifying tissues such as tracheids, sclereids and hypodermal sclerenchyma. This indicates that mannan, as it is the case in gymnosperms, is a major constituent of secondary cell walls. Indeed, we detected high levels of mannan epitopes in both the NaOH and CDTA cell wall extractions of isolated sclerenchyma of *A. elliotii* and generally the levels of mannan epitopes reflected the relative amount of secondary cell walls in the ferns and lycophytes samples. As the LM21 epitope was restricted to the inner cell wall layers of secondary cell walls, it is possible that these epitopes might be masked as a result of the incorporation of autofluorescent phenolic compounds. In our CoMPP-analysis we did not find any taxonomically significant differences in the amount of mannan-epitopes in petioles of lycophytes, and eusporangiate and leptosporangiate ferns.

Glycan epitopes of arabinogalactan-proteins show (taxonomically) restricted distribution patterns

The occurrence of the AGP LM2 epitope is of taxonomical significance as we showed, both in our CoMMP analysis and IC experiments, that this epitope is restricted to homosporous lycophytes. It is of interest that the LM2 epitope was detected in NaOH extracts whereas the JIM4 and JIM13 AGP epitopes were most abundant in the CDTA extracts of the non-lycophytes where they were widely detected. None of the AGP-antibodies bound to cell walls. In some cases AGP-labelling of parenchymatous cell types of the vascular bundles corresponded with the LM6-labelling of cell walls of the same tissue types.

7.5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

We are still in the early stages of exploring cell wall diversity within the plant kingdom and this study emphasizes that several elements of cell wall polysaccharides have taxonomically significant distribution patterns and that groups of plants can have specific configurations of polymers within walls of specific cell-types or tissues.

The presence and/or *in situ* distribution patterns of some epitopes showed clear differences at different taxonomical levels: between the major groups of ferns and lycophytes studied (homosporous and heterosporous lycophytes, eusporangiate, and leptosporangiate ferns – LM2, LM5, LM6, HG-epitopes), as well as within the leptosporangiate ferns (CCRC-M1) and the Aspleniaceae (LM6).

The major novel observation that has arisen during this analysis is that many cell wall epitopes are clearly associated with specific tissues, cell types or cell wall modifications (e.g. LM5, LM6, LM15, LM11, LM21), and this confirms that functional specialisation of each of these cell types and tissues is indeed reflected in the molecular design of the cell wall. Therefore, future studies should focus on individual cell walls rather than organs.

A future challenge will be to integrate phylogenetic reconstruction, morphological studies and developmental genetic data in order to understand how specific configurations of polysaccharides and their interactions underpin cell wall functions. To better understand the structure-function relationships of specific cell walls, micromechanical and microstructural characterisations at tissue or cell-type level (Burgert, 2006; Sarkar *et al.*, 2009; Lee *et al.*, 2011) will be required to be combined with molecular knowledge.

7.6 ACKNOWLEDGEMENTS

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Appendix 1: List of studied material with voucher information. BGUG: Botanical Garden University of Ghent.

- Actiniopteris australis*: Réunion; VIANE 8433.
Adiantum capillus-veneris: Location unknown; BGUG.
Angiopteris hypoleuca: Location unknown; GUBG 1967/0852.
Antrophyum giganteum: Réunion; VIANE 8268.
Arachniodes aristata: Location unknown; BGUG.
Asplenium aethiopicum **spp. aethiopicum**: Uganda; VIANE 11230.
Asplenium affine: Réunion; VIANE 8227.
Asplenium auriculatum: Puerto Rico; VIANE 10602.
Asplenium auritum: Venezuela; VIANE 10256.
Asplenium boltonii: Réunion; VIANE 6403.
Asplenium centrafricanum: Uganda; BELLEFROID 392.
Asplenium compressum: St. Helena; VIANE 8851.
Asplenium cristatum: Puerto Rico; VIANE 10651.
Asplenium currorii: Ivory Coast; GUBG 1979/2824.
Asplenium cuspidatum: Venezuela; VIANE 10112.
Asplenium daucifolium **ssp. viviparum**: Réunion; VIANE 8445.
Asplenium dregeanum: Uganda; VIANE 11199.
Asplenium exiguum: Mexico; VIANE 11102.
Asplenium friesiorum: Uganda; VIANE 11183.
Asplenium hemionitis: Location unknown; BGUG.
Asplenium javorkeanum: CV405.
Asplenium juglandifolium: Puerto Rico; VIANE 10667.
Asplenium loxoscaphoides: Uganda; BELLEFROID 340.
Asplenium lunulatum: Rep. South Africa; VIANE 6558.
Asplenium nidus s.l.: Myanmar; VIANE 9630.
Asplenium polyodon: Uganda; VIANE 11211.
Asplenium radicans: Puerto Rico; VIANE 10639.
Asplenium ruta-muraria: France; VIANE 10040.
Asplenium rutifolium: Uganda; VIANE 11510.
Asplenium scolopendrium: Belgium; VIANE 10971.
Asplenium serra: Venezuela; VIANE 10222.
Asplenium sertularioides: Uganda; BELLEFROID 405.
Asplenium tenerum: Indonesia; VIANE 9043.
Asplenium tenuicaule: China; VIANE 10888.
Asplenium theciferum: Kenya; BELLEFROID 297.
Asplenium trichomanes: France; VIANE 9566.
Asplenium varians **ssp. varians**: China; VIANE 10733.
Asplenium vulcanicum: Indonesia; NJO 35.
Athyrium filix-femina: Location unknown; BGUG.
Blechnum brasiliense: Location unknown; BGUG.
Blotiella pubescens: Réunion; VIANE 8226.
Bolbitis heteroclita: Genova; BGUG 1970/0086.

Cheilanthes sp.: Mexico; VIANE 11051.
Cyathea capensis: Rep. South Africa; VIANE 6615.
Cystopteris cf. millefolia: Mexico; VIANE 11079.
Danaea elliptica: Puerto Rico; VIANE 10636.
Davallia trichomanoides: Location unknown; BGUG 1970/0085.
Diplazium proliferum: Réunion; VIANE 8281.
Drynaria cf. mollis: China; VIANE 6985.
Dryopteris intermedia ssp. maderensis: Portugal; VIANE 8940.
Elaphoglossum sp.: Tanzania; VIANE 7718.
Equisetum arvense: Location unknown; BGUG.
Equisetum hyemale: Location unknown; BGUG.
Equisetum ramosissimum: La Palma; VIANE 8118.
Equisetum x littorale: Location unknown; BGUG.
Hymenasplenium excisum: China; VIANE 11263.
Hymenasplenium obscurum: China; VIANE 11286.
Hymenasplenium unilaterale: Réunion; VIANE 8334.
Lepisorus sp.: Uganda; VIANE 11233.
Lycopodium gnidioides: Réunion; VIANE 8481.
Lycopodium squarrosum: Location unknown; BGUG.
Nephrolepis hirsutula: Location unknown; BGUG.
Osmunda vauchelii: China; VIANE 11306.
Pellaea falcata: Location unknown; BGUG 1975/1278.
Platyserium bifurcatum: Location unknown; BGUG.
Polypodium azoricum: Portugal; VIANE 5394.
Polystichum sp.: Venezuela; VIANE 10134.
Psilotum nudum: Location unknown; BGUG.
Pteridium aquilinum: Location unknown; BGUG.
Pteris cretica: Réunion; VIANE 8475.
Pyrrosia sp.: Uganda; VIANE 11223.
Rumohra sp.: Venezuela; VIANE 10104.
Salvinia auriculata: Location unknown; BGUG.
Selaginella grandis: Location unknown; BGUG.
Selaginella kraussiana: Rep. South Africa; VIANE 6566.
Tectaria gemmifera: Kenya; BGUG 75/3306.
Thelypteris sp.: Réunion; VIANE 8261.
Todea barbara: Rep. South Africa; VIANE 11588.
Trichomanes speciosum: Spain; VIANE 5520.
Woodwardia orientalis: Location unknown; BGUG 1900/2729.

8

An extensin-rich matrix lines the carinal canals in *Equisetum ramosissimum* which may function as water-conducting channels

This chapter is a modified version of:

Leroux O., Knox J.P., Masschaele B., Bagniewska-Zadworna A., Marcus S.E., Claeys M., van Hoorebeke L., Viane R.L.L. A specialised cell wall lines the carinal canals in *Equisetum ramosissimum* Desf. (Equisetaceae, Pteridophyta) (Annals of Botany, *in press*)

Summary

The anatomy of *Equisetum* stems is characterized by the occurrence of vallecular and carinal canals. Previous studies on the carinal canals in several *Equisetum* species suggest that they convey water from one node to another. Cell wall composition and ultrastructure have been studied using immunocytochemistry and electron microscopy respectively. Serial sectioning and X-ray computed tomography were employed to examine the internode-node-internode transition of *Equisetum ramosissimum*. The distribution of the LM1 and JIM20 extensin epitopes is restricted to the lining of carinal canals. The monoclonal antibodies JIM5 and LM19 directed against homogalacturonan with a low degree of methyl-esterification and the CBM3a probe recognizing crystalline cellulose also bound to this lining. The xyloglucan epitopes recognized by LM15 and CCRC-M1 were only detected in this lining after pectate lyase treatment. The carinal canals, connecting consecutive rings of nodal xylem, are formed by the disruption and dissolution of protoxylem elements during elongation of the internodes. Their inner surface appears smooth compared to that of vallecular canals. In conclusion, the carinal canals in *Equisetum ramosissimum* have a distinctive lining containing pectic homogalacturonan, cellulose, xyloglucan and extensin. These canals might function as water-conducting channels which would be especially important during the elongation of the internodes when protoxylem is disrupted and the metaxylem is not yet differentiated. How the molecularly-distinct lining relates to the proposed water-conducting function of the carinal canals requires further study. Efforts to elucidate the spatial and temporal distribution of cell wall polymers in a taxonomically broad range of plants will likely provide more insight into the structural-functional relationships of individual cell wall components or of specific configurations of cell wall polymers.

8.1 INTRODUCTION

Equisetum is a genus of approximately 15 to 25 extant hollow stemmed taxa (Milde, 1867; Sadebeck, 1902; Smith, 1955; Hauke, 1963, 1978, 1990; Guillon, 2007) which are the only link to a group of extinct, diverse and dominant pteridophytes (Brongniart, 1828; Frank, 1877; Eames, 1936; Hirmer, 1938; Emberger, 1944; Stewart, 1983; Bateman, 1991; Rothwell, 1996; Stanich *et al.* 2009). In the last two decades there has been an increased interest in the phylogeny (Des Marais *et al.*, 2003; Guillon, 2004; Guillon, 2007), biomechanics (Speck *et al.*, 1998; Spatz *et al.*, 1998; Spatz and Emanns, 2004) and cell wall biology (Popper and Fry, 2004; Gierlinger *et al.*, 2008; Fry *et al.*, 2008; Sørensen *et al.*, 2008; Currie and Perry, 2009) of this remarkable genus.

Recent phylogenetic studies (Kenrick and Crane, 1997; Pryer *et al.*, 2001; Qiu *et al.*, 2007; Karol *et al.*, 2010) separate Lycophyta from the core pteridophytes, and unite Equisetaceae to the true ferns (Pteridophyta s.s.) as a monophyletic group. In recent cell wall research, *Equisetum* has received a lot of attention as mixed-linkage (1→3)(1→4)-β-D-glucan (MLG), previously thought only to occur in Poales cell walls, was found abundantly in *Equisetum* cell walls (Sørensen *et al.*, 2008; Fry *et al.*, 2008). The discovery of this polysaccharide in a land plant outside Poales suggested novel cell wall architectures unlike either type I or type II cell walls (Knox, 2008).

The general morphology as well as the anatomy of the mature stem, first described in detail by Bischoff (1828), Brongniart (1828), and Milde (1865), is so characteristic that the genus has been distinguished correctly from other superficially similar plant taxa since Tournefort (1719, see Milde 1867 for a review). A transverse section of the internode shows a pattern of subepidermal strengthening tissue and chlorenchyma, vallecular canals in the cortex opposite the furrows, and carinal canals opposite the ridges. The vascular bundles are always positioned opposite a ridge, forming a ring surrounding the central cavity (Ogura, 1972). Westmaier (1884) was the first to suggest that carinal canals, which are protoxylem lacunae, are water-conducting canals. This was later accepted by Sykes (1906), Schaffner (1908), Barrat (1920), and Bierhorst (1958a). Brongniart (1828) and Cormack (1893) pointed out that the carinal canals are not continuous from one internode to the next, and that nodal xylem elements convey water from one canal to another. These nodal xylem elements were later studied by Sykes (1906) and Bierhorst (1958a, b).

In light of the recent discovery of mixed-linkage glucans in *Equisetum* cell walls (Sørensen *et al.*, 2008; Fry *et al.*, 2008), and the observation of complex distribution patterns of cell wall epitopes during a comparative study of fern cell walls, we decided to perform a detailed study focusing on the distribution patterns of cell wall epitopes in

Equisetum stems. Our immunocytochemical study revealed a restricted occurrence of extensin epitopes to the carinal canal linings. Therefore we also re-examined the ontogeny and structure of these canals with attention given to the nodal anatomy, as this has poorly been documented in the recent literature.

8.2 MATERIAL AND METHODS

8.2.1 Plant material

Plant material of *Equisetum ramosissimum* was collected in Spain (La Palma, Caldera de Taburiente, Galeria Aridane, 1100 m, Viane 8118) and kept in our living collection at the Ghent Botanical Garden, Belgium.

8.2.2 Histology

Internodal and nodal segments of mature and young *Equisetum* stems were fixed overnight in FAA (50% v/v ethanol, 5% v/v acetic acid and 5% v/v commercial formalin in distilled water), dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) following Leroux *et al.* (2007a [CHAPTER 2]). Transverse sections of 4 µm were cut with a Microm HM360 microtome (Microm International GmbH, Walldorf, Germany) equipped with a holder for glass knives, dried on Vectabond-coated (Vector Laboratories, Burlingame, CA, USA) slides, stained with an aqueous 0.05% w/v solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% w/v Na₂B₄O₇ (pH 8.5) and mounted in DePeX (Gurr, BDH Laboratory, UK). Phloroglucinol/HCl staining was performed on fresh hand-cut sections using 2% w/v phloroglucinol in 95% v/v ethanol for 5 min, and subsequently mounting in 33% v/v hydrochloric acid. For MaÛle staining, fresh hand-cut sections were immersed in 1% (w/v) potassium permanganate for 10 min at room temperature and then washed twice with 3% hydrochloric acid. Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

8.2.3 X-ray Computed Tomography and drawings

Samples fixed in FAA and processed following Leroux *et al.* (2009 [CHAPTER 3]) were scanned at the centre for computed tomography (UGCT) of Ghent University (Masschaele *et al.*, 2007). For the measurements we used an in-house scanner the main components of which are: an open type Hamamatsu nanofocus X-ray tube (L10711); a second sealed type Hamamatsu microfocus (L9181-02); a high resolution CCD camera

with fiber optic taper from the company Photonic Science: a Varian amorphous silicon flat panel detector (Paxscan 2520 with CsI scintillator), and a sample manipulator which can move the samples with 5 degrees of freedom. The samples were scanned with the L9181 tube at 70 kV and 60 μ A in order to obtain high contrast and high spatial resolution. The samples were rotated from 0° to 360° in steps of 0.45°. The Varian detector was set to an exposure time of 400 ms and 6 frames were averaged per rotation step to increase the statistics of the images. The samples were magnified 42 times in order to reach a voxel size of 3 μ m in the CT images. The raw projection images were processed into CT slices with Octopus. Octopus is a user friendly, system independent, reconstruction package developed and commercialized by Ghent University (www.inct.be). After the reconstruction we obtained over 800 cross sections through the sample volume. The software programme VGStudio Max (<http://www.volumegraphics.com>) was used to create the 3D renderings. Drawings were based on a selection of virtual cross sections and were made with CorelDRAW Graphics Suite 12.

8.2.4 Transmission electron microscopy

Equisetum stems were dissected in order to produce blocks measuring approximately 2 mm length at all sides. They were fixed with 2% w/v paraformaldehyde and 2% v/v glutaraldehyde in a cacodylate buffer 0.1 M pH 6.9 for 24 h at 4°C, post fixed in 2% osmium tetroxide for 3 h, washed in the same buffer and dehydrated in a step gradient of ethanol. The samples were transferred to 100% alcohol/Spurr's resin (1:1) at 4°C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4°C), and transferred to 100% Spurr's resin and left overnight at 4°C. Polymerization was performed at 70°C for 16 h. Seventy-nm-thick sections were made using a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria). Formvar-coated single slot copper grids were used. Sections were stained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 10 min in lead citrate stain at 20°C. The grids were examined with a JEM-1010 electron microscope (Jeol Ltd, Tokyo, Japan) equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

8.2.5 Scanning electron microscopy

Longitudinal hand-cut root sections were fixed in FAA, dehydrated in a graded ethanol series followed by a graded acetone series, and dried in a critical point dryer (Balzers CPD-030) using CO₂ as a transition fluid. Dried sections were mounted on clean aluminum stubs with double-sided adhesive graphite tabs. Mounted specimens were

coated with gold (12–15 nm thick) using Balzers SPD-050 sputter coater. Sections were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

8.2.6 Immunocytochemistry

For indirect immunofluorescence labelling, hand-cut sections of internodal segments were made. A range of highly specific cell wall-directed probes available was used to evaluate the presence of major cell wall polymers including pectins, xyloglucans, (glucogalacto)mannans, (arabino)xylans, and extensins (see Table 8.1).

The sections were incubated in 5% w/v milk protein in PBS (MP/PBS) for 5 min to block non-specific binding sites. Sections were then incubated with primary rat monoclonal antibodies (JIM5, JIM7, LM19, LM20, LM5, LM6, LM13, LM16, LM10, LM11, LM15, LM21, LM1, JIM20; for references see table 1) diluted 1:10 in MP/PBS for at least 1 h. The sections were washed with several changes of PBS prior to incubation with the secondary antibody, anti-rat-IgG linked to fluorescein isothiocyanate (FITC; Sigma) diluted 1:100 in 5% w/v MP/PBS for 1 h. CCRC-M1, a monoclonal antibody developed in mice (Puhlmann *et al.*, 1994) was used in 1:5 dilutions and binding was visualized with a 50-fold diluted mouse anti-his monoclonal antibody (Sigma) in MP/PBS for 1.5 h. The binding of CBM3a (Blake *et al.*, 2006) was assessed by a three-stage immunolabeling procedure. After incubating in MP/PBS containing 40 µg/ml of CBM3a protein for 1.5 h, sections were washed in PBS at least three times and incubated with a 100-fold dilution of mouse anti-his monoclonal antibody (Sigma) in MP/PBS for 1.5 h. Following washing with PBS, anti-mouse-IgG linked to fluorescein isothiocyanate (FITC; Sigma) was applied for 1.5 h as a 50-fold dilution in MP/PBS. Labelling with the Biosupplies 400-3 anti-mixed-linkage glucan antibody was performed as described elsewhere (Meikle *et al.*, 1994). Cellulose was stained with Calcofluor White M2R fluorochrome (fluorescent brightener 28, Sigma, 0.25 µg mL⁻¹ in dH₂O). Subsequently, all sections were washed in PBS buffer three times before mounting in a glycerol-based anti-fade solution (Citifluor AF1, Citifluor Ltd., UK). Recent studies showed that cell wall epitopes can be masked and where appropriate we performed pectate lyase treatments as described by Marcus *et al.* (2008). Immunofluorescence was observed with a microscope equipped with epifluorescence irradiation (Olympus BX-61) and images were captured with a Hamamatsu ORCA285 camera and prepared with Improvision Volocity software.

8.2.7 Peroxidase assay

In-situ peroxidase activity was detected by treatment of fresh hand-cut sections in 5 mg mL⁻¹ 3,3'-diaminobenzidine (DAB)-HCL, pH 3.8, re-buffered to pH 5.8 immediately before use. Subsequently 1 mM H₂O₂ was added and incubation was performed at room temperature for 5 minutes.

CBM27 ⁽¹⁰⁾	mannan	+	-	+	+	+	+	-	+	+	+	+ ^s	+	-
CBM3a ⁽¹¹⁾	crystalline cellulose	+	+	+	+	+	+	++	+	++	++	+	+	+
Biosupplies 400-2 ⁽¹²⁾	(1→3)-β-glucan	-	-	-	-	-	-	-	-	-	-	-	+ [†]	-
LM1 ⁽¹³⁾	extensin	-	-(±)	-	-	-	-(±)	+	-	-	-	-	-	-
JIM20 ⁽¹⁴⁾	extensin	-	±	-	-	-	-	+	-	-	-	-	-	-

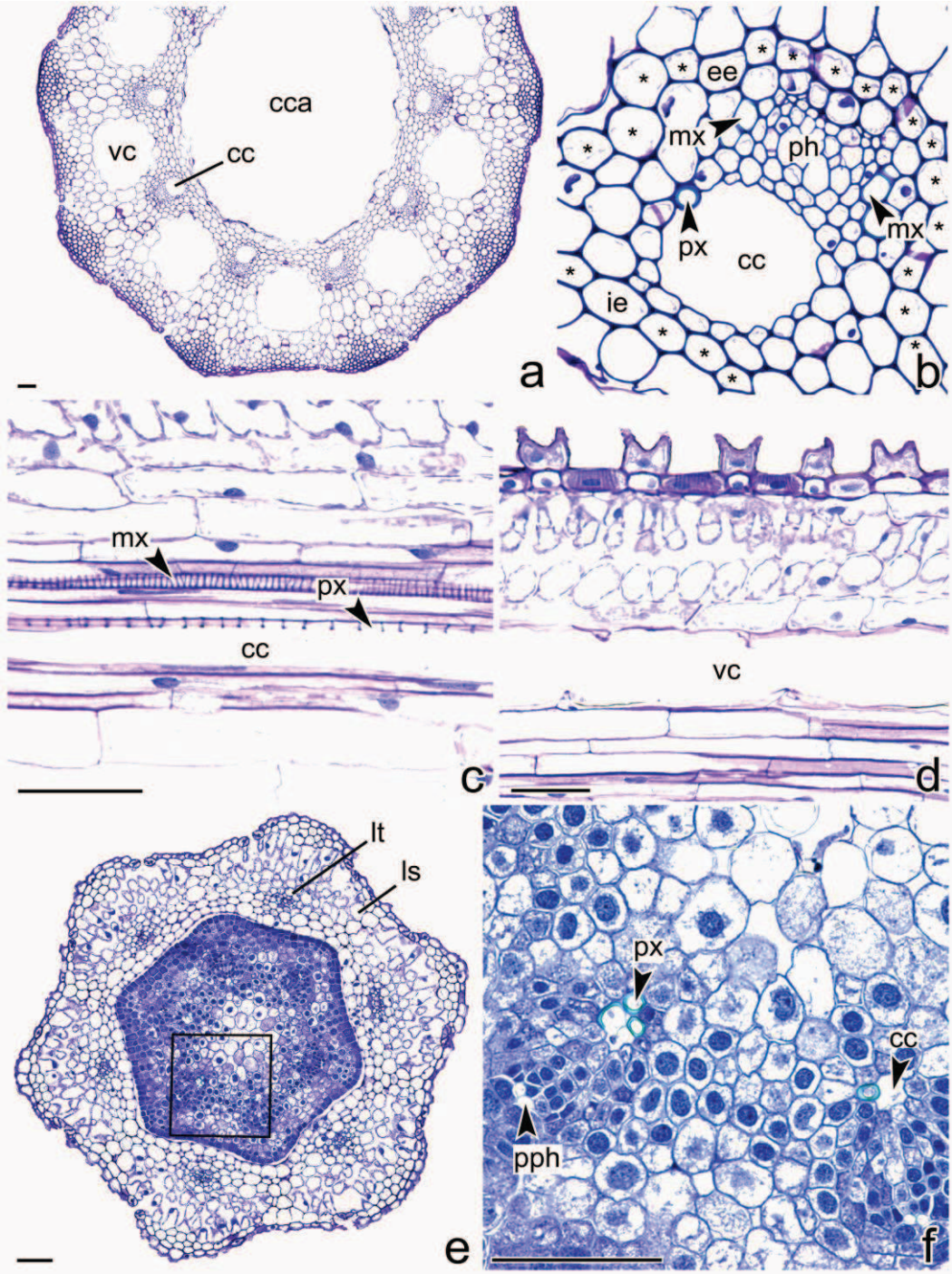
References: ¹Clausen *et al.* (2003), ²Verhertbruggen *et al.* (2009b), ³Jones *et al.* (1997), ⁴Willats *et al.* (1998), ⁵Verhertbruggen *et al.* (2009a), ⁶McCartney *et al.* (2005), ⁷Marcus *et al.* (2008), ⁸Meikle *et al.* (1994), ⁹Puhlmann *et al.* (1994), ¹⁰Marcus *et al.* (2010), ¹¹Blake *et al.* (2006), ¹²Meikle *et al.* (1991), ¹³Smallwood *et al.* (1995), ¹⁴Smallwood *et al.* (1994).

8.3 RESULTS

8.3.1 Anatomy of the internode

Cross-sections through mature internodes of *E. ramosissimum* show the typical internodal anatomy described in the introduction (Fig. 8.1a), characterized by strengthening tissues in the ridges, carinal (Fig. 8.1a, b, c) and vallecular (Fig. 8.1a, d) canals as well as a central cavity occupying nearly half of the total diameter (Fig. 8.1a). The vascular bundles are enclosed by a common internal and external endodermis (Fig. 8.1b). In each vascular bundle two groups of xylem elements, which will further be referred to as metaxylem, are positioned laterally at both sides of the phloem (Fig. 8.1b). In longitudinal sections of mature internodes the lining of the carinal canals is smooth apart from occasional protoxylem rings (Fig. 8.1c), whereas in vallecular canals remnants of disrupted parenchyma cells are visible (Fig. 8.1d). In older internodes, the parenchyma cell walls facing the carinal canals are slightly thickened. Figure 1e shows a transverse section of a young internode surrounded by a leaf sheath, showing six developing vascular bundles, but without fully developed carinal or vallecular canals. A detail of a vascular bundle (Fig. 8.1f) shows both protophloem and protoxylem, as well as some very small carinal canals, which developed after disruption and/or dissolution of protoxylem elements. Vallecular canals and central cavities were seen to develop through disruption of cortex and pith parenchyma respectively, prior to the differentiation of the metaphloem and metaxylem (data not shown). In all young internodes studied, the central cavities developed prior to the vallecular canals, and metaxylem differentiated after the elongation of the internodes had ceased (data not shown).

Figure 8.1: Transverse (a, b, e, f) and longitudinal (c, d) sections through mature (a–d) and young (e, f) internodes of *Equisetum ramosissimum*. **a.** Internode showing central cavity (cca), carinal (cc) and vallecular (vc) canals. **b.** Vascular bundle consisting of a carinal canal (cc) with protoxylem (px) elements, metaphloem (ph) and metaxylem elements (mx). Ring of vascular bundles enclosed by an internal (ie) and external (ee) endodermis. Endodermis cells are indicated with asterisks. **c.** Part of vascular bundle showing carinal canal (cc) with smooth surface, protoxylem (px) with annular thickenings, and scalariform metaxylem (mx). **d.** Epidermis with silicifying papillae and cortex with vallecular canals (vc) with rough surface caused by disintegrated cells. **e.** Young internode surrounded by leaf sheath (ls) with leaf traces (lt). Protoxylem starting to disintegrate and forming small carinal canals. **f.** Detail (indicated in figure 1e) of differentiating vascular bundle with protophloem (pph) and protoxylem (px). Metaxylem and metaphloem yet undifferentiated. Scalebars: 100 μ m.



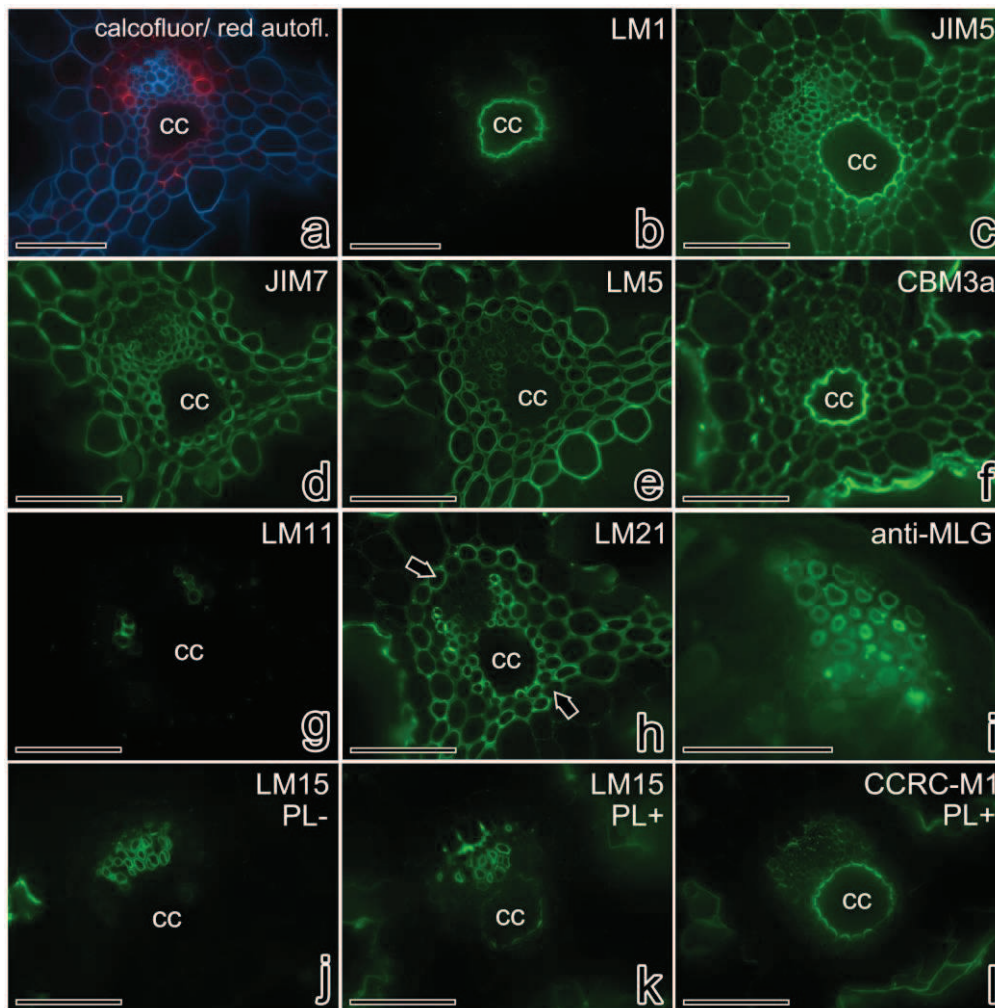


Figure 8.2: Immunolabelling of transverse sections through *E. ramosissimum* internodes using several probes directed against different cell wall epitopes. **a.** Combined image of calcofluor staining (blue), and red autofluorescence of Casparian strips. **b.** LM1 epitope (extensin) restricted to lining of carinal canals, same section as in **a**. **c.** Binding of JIM5 (pectic homogalacturonan with high levels of esterification) to lining of carinal canals and middle lamellae of parenchymatous cells. **d.** JIM7 epitope (pectic homogalacturonan with low levels of esterification) occurring in primary cell walls but absent from carinal canal lining. **e.** Binding pattern of LM5 similar to that of JIM7. **f.** CBM3a binding to all cell walls as well as to lining of carinal canals. **g.** LM11 (anti-xylan) binding to metaxylem elements. **h.** LM21 mannan epitopes detected in walls of endodermis (arrows) and enclosed pericycle and parenchyma, and in lining of vallicular canals. **i.** Anti-MLG antibody binding to inner cell wall regions of strengthening tissue. **j.** LM15 strongly binding to phloem cell walls, but weakly to lining of vallicular canals. **k, l.** Effect of pectate lyase treatment showing LM15 (**k**) and CCRC-M1 (**l**) binding to lining of carinal canals. cc: carinal canals. Scale bars: 100 μ m.

8.3.2 Immunofluorescence labelling of cell wall components and lignin staining

We used several monoclonal antibodies and a carbohydrate-binding module to examine the presence of major cell wall constituents including pectins, cellulose, hemicelluloses and extensins in *Equisetum ramosissimum* stems (Fig. 8.2). Results are summarized in Table 1 and relevant and specific binding patterns are discussed here. Figure 8.2a is a combined image of Calcofluor staining and red autofluorescence. Calcofluor, with a high affinity for (1→4)-β-D-glucans and (1→3)-β-D-glucans, stained all cell walls, except metaxylem secondary cell walls, Casparian strips, and the lining of the carinal canals. Guard cells as well as phloem cells (Fig. 8.2a) were intensively stained. Red autofluorescence of Casparian strips of both the internal and external endodermis and of the metaxylem secondary cell walls was evident (Fig. 8.2a). The source of this autofluorescence is, however, unclear. The LM1 and JIM20 antibodies, both recognizing extensin epitopes, bound to the lining of the carinal canals (Fig. 8.2b). Extensin-epitopes are not restricted to the lining of carinal canals, as pectate lyase treatment revealed a very weak binding of LM1 to cell wall corners of the cortex parenchyma (data not shown). The monoclonal antibodies JIM5 and LM19, which bind to homogalacturonan with a low degree of methyl esterification, clearly labelled the lining of the carinal canals as well as most parenchymatous cell walls in the stem (Fig. 8.2c). Binding of JIM5 was strongest at cell wall corners and to the middle lamellae, whereas it bound relatively weakly to primary cell walls. In contrast, JIM7 and LM20, recognizing homogalacturonan with a high degree of methyl esterification, bound to all primary cell walls but not to the lining of the carinal canal (Fig. 8.2d). LM5, binding to (1→4)-β-D-galactan, labelled all parenchymatous cell walls in a similar way to JIM7 (Fig. 8.2e). The (1→5)-α-L-arabinan epitope, recognized by LM6, occurred in all parenchyma cell walls as well as in subsidiary and guard cell walls. Two other anti-arabinan antibodies, LM13 and LM16, bound, respectively, to the cell walls of subsidiary and guard cells of the stomatal complex of *Equisetum ramosissimum* confirming the results shown by Verhertbruggen *et al.* (2009a). CBM3a, a carbohydrate-binding module which recognizes crystalline cellulose, bound to all cell walls, including the lining of the carinal canals (Fig. 8.2f). Binding of the anti-xylan antibodies LM10 and LM11 was restricted to the secondary cell walls of the scalariform metaxylem tracheids (Fig. 8.2g) and to the guard cell walls. Mannan-epitopes, recognized by LM21, were especially abundant in all parenchymatous cell walls enclosed by the inner and outer endodermis (Fig. 8.2h), and those of the epidermis and strengthening tissue. Pretreatment with pectate lyase, showed the presence of the LM21 epitope in all cell walls, including those of the phloem. An anti-mixed-linkage glucan antibody only bound to the inner wall regions of the cells of the

strengthening tissue (Fig. 8.2i) and to the walls of some cortical parenchyma cells located between the chlorenchyma and the vallecular canals. It is of interest that LM15, an antibody binding to the XXXG motif of xyloglucan, strongly bound to sieve cells of the phloem (Fig. 8.2j) and to guard cells, but weakly to the lining of vallecular canals and the central cavity. However, LM15 reacted weakly with the lining of the carinal canals after pectate lyase treatment (Fig. 8.2k). CCRC-M1, which recognizes fucosylated xyloglucan bound to the phloem cell walls and strengthening tissue, and to the lining of vallecular and central canals. Pectate lyase treatment unmasked this epitope in the lining of carinal canals (Fig. 8.2l).

In order to assess the presence of lignins, we stained fresh hand-cut sections with Wiesner (Fig. 8.3a-d) and Maüle reagent. These stains both gave the same result showing the presence of lignin in the xylem tracheids and Casparian strips, and its absence in the cell walls of the strengthening tissue (Fig 8.3a, c). We performed a peroxidase activity assay and, in the presence of exogenous hydrogen peroxidase, DAB strongly stained the cell walls of the endodermis, epidermis, the cortical parenchyma, the metaxylem, as well as the lining of the carinal canals (Fig. 8.3e). The cell walls of the remaining tissues, including the phloem, pericycle, and the parenchyma enclosed by the endodermis, and surrounding the vallecular canals and the central cavity, were not stained (Fig. 8.3e).

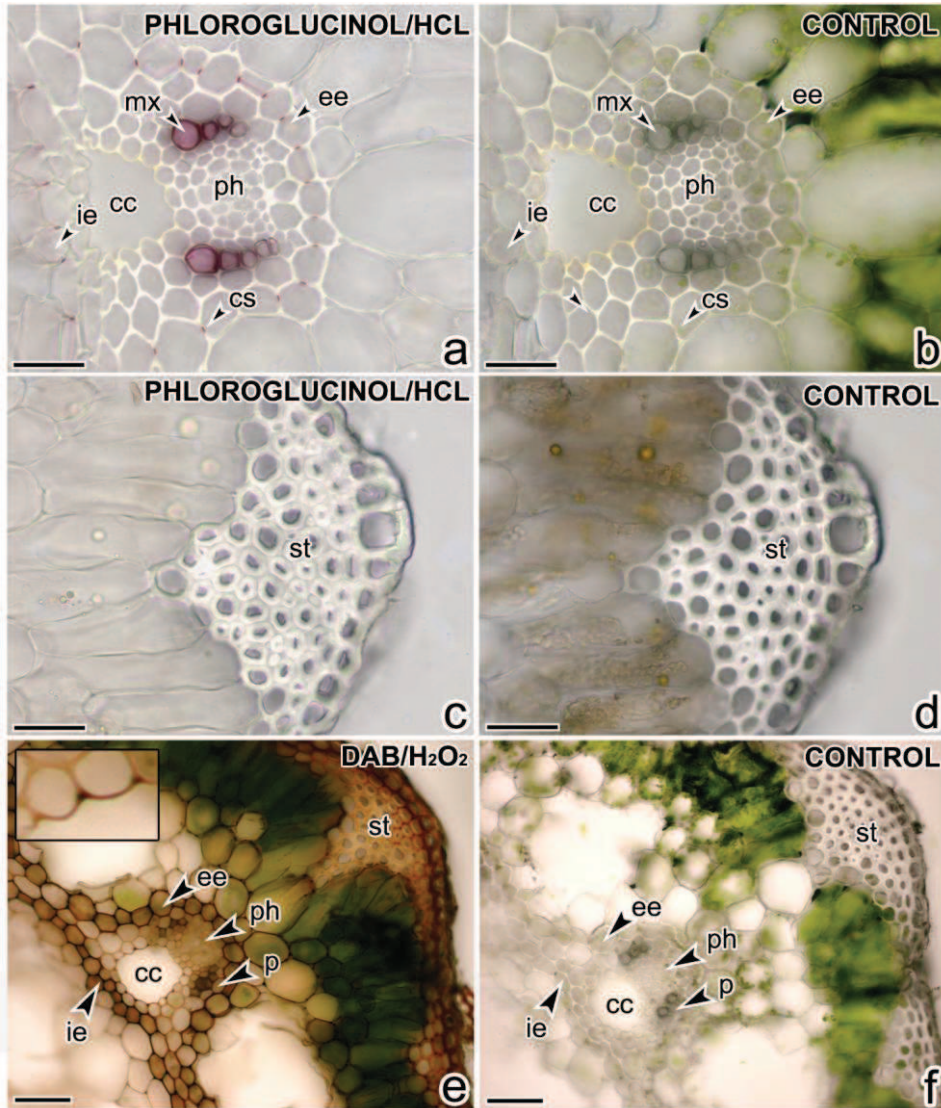


Figure 8.3: Localisation of lignins through phloroglucinol/HCl-staining (a-d) and determination of peroxidase activity using DAB/hydrogen peroxide (e-f) in transverse sections of *E. ramosissimum* stems. **a, b.** Walls of the metaxylem tracheids and Casparian strips of internal (ie) and external endodermis (ee) intensely red suggesting presence of lignins. **c, d.** Walls of the strengthening tissue uncoloured suggesting absence of lignins. **e, f.** Walls of epidermis, cortical parenchyma, internal (ie) and external endodermis (ee), and lining of carinal canal (inset) staining blackish-brown, whereas phloem (ph), pericycle (p), strengthening tissue (st), parenchyma enclosed by double endodermis, and parenchyma surrounding vallicular canals and central cavity remaining unstained.

8.3.3 Electron microscopy

Transmission electron microscopy showed the absence of intercellular spaces in the tissues enclosed by the internal and external endodermis. Instead, these spaces (Fig. 8.4a) are filled with intensely stained materials, comparable to that of the middle lamellae. A similar material also lines the carinal canals (Fig. 8.4a, b). A detail of the border of a vallecular canal shows cell wall remnants of disrupted parenchyma cells (Fig. 8.4c); an electron dense coating similar to that present in the carinal canals was not found. The surface of the carinal and vallecular canals, as well as that of the central cavities was also studied using scanning electron microscopy. Carinal canals have a smooth surface, (Fig. 8.4d) occasionally with attached protoxylem rings (not shown). In contrast, both the central cavity (Fig. 8.4e) and the vallecular canal (Fig. 8.4f) are characterized by a rough surface. Xylem elements in the nodes are short and show multiseriate pitting (Fig. 8.4g) whereas the internodal metaxylem elements have a scalariform thickening (Fig. 8.4h).

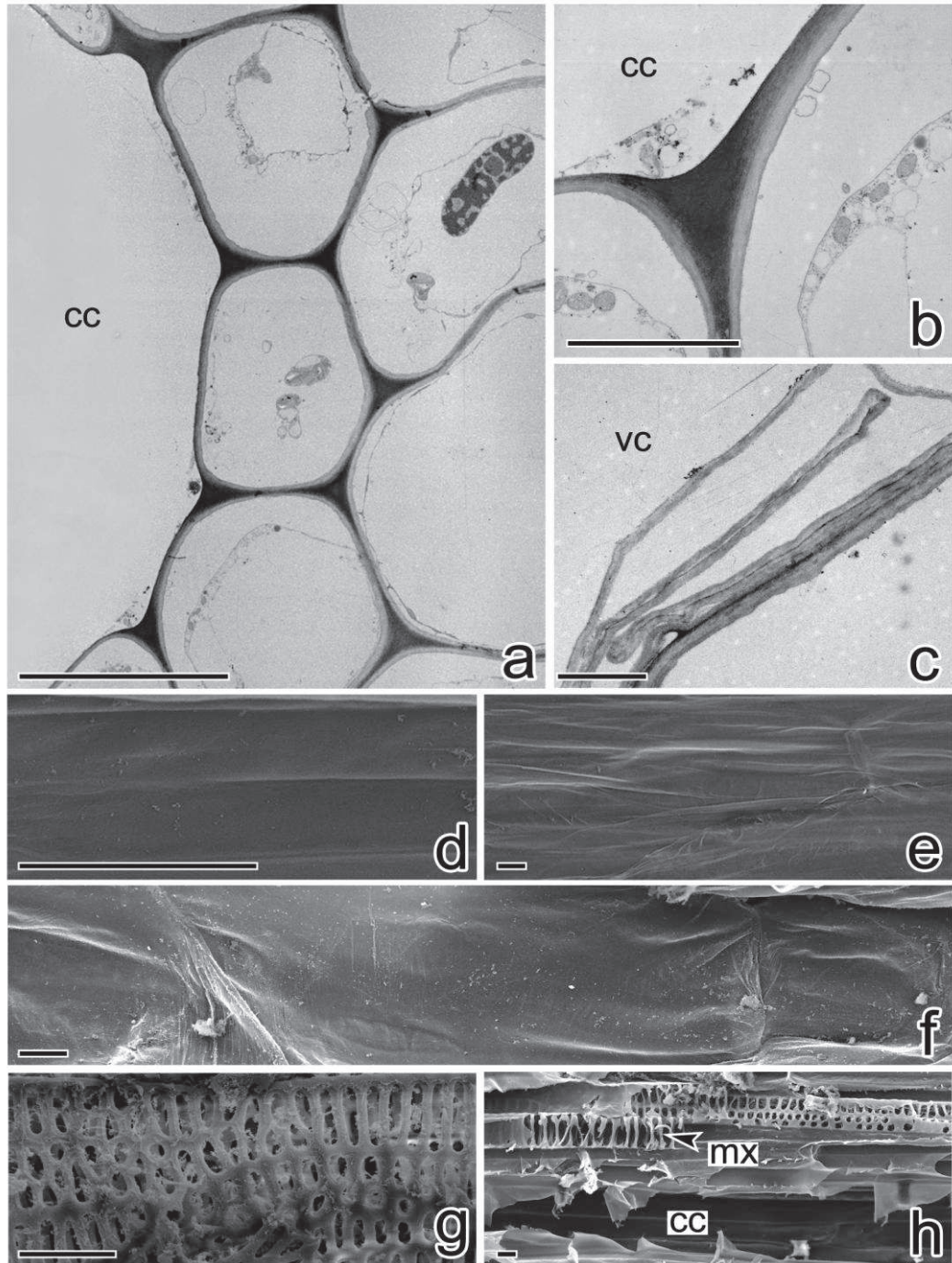
8.3.4 Nodal interconnection of carinal canals

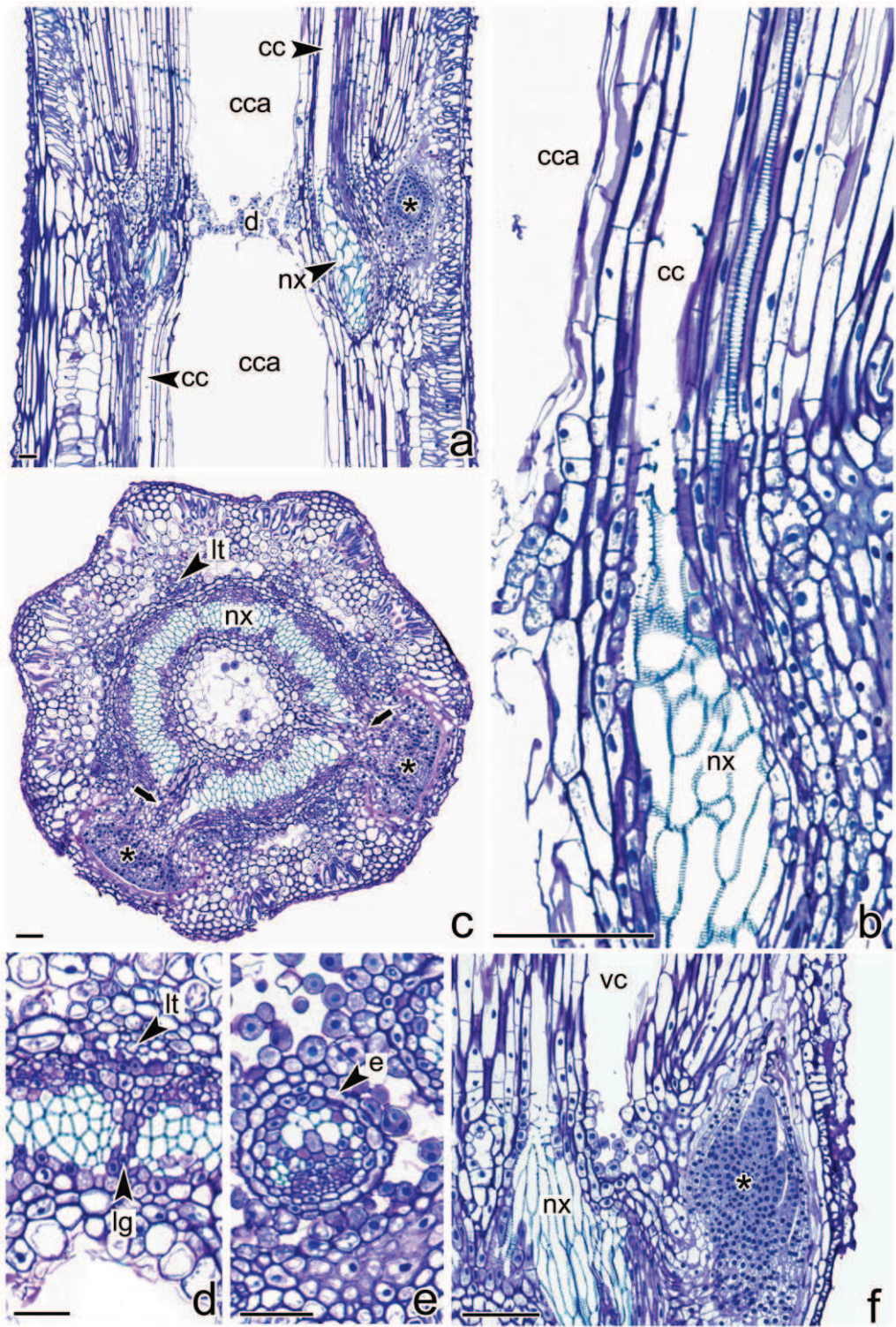
In the node, leaf and branch traces depart and the position of vascular bundles of successive internodes changes. Nodes show a different arrangement of xylem and phloem elements compared to the internode (Fig. 8.1a, 8.5c), and have an ectophloic siphonostele (Fig. 8.5c) with leaf gaps as well as branch gaps. Longitudinal median sections (Fig. 8.5a) show the diaphragm which forms an interruption between the central cavities of consecutive internodes. Furthermore, the vallecular canals are completely interrupted in the node (Fig. 8.5c, f), while the carinal canals are connected to the nodal xylem (Fig. 8.5a, b).

Good-quality serial sectioning – without the loss of several sections – of *Equisetum* nodes was difficult due to the high silica content of the cell walls. The internode-node-internode transition in *E. ramosissimum* was therefore also studied by X-ray computed tomography (Fig. 8.6). In addition, this method allowed the generation of perfectly aligned serial virtual sections in both transverse and longitudinal planes. The schematic drawings in figure 8.7 were largely based on these aligned virtual sections. In each vascular bundle the nodal transition starts with the gradual increase of xylem elements towards the carinal canals (Fig. 8.7b). Closer to the node, the carinal canals are gradually replaced by xylem elements (Fig. 8.7c), which bulge out and first form branch traces later followed by leaf traces (Fig. 8.7c, d). In contrast to other *Equisetum* species, where branches are formed between all leaf traces (Bischoff, 1828, 1834; Stenzel, 1861; Gwynne-Vaughan, 1901; Browne, 1912), branches develop irregularly in *E. ramosissimum*.

Leaf traces are formed opposite each ridge and in the prolongation of the original vascular bundle (Fig. 8.7d). The departing leaf traces are clearly associated with narrow leaf gaps as shown in figure 8.5d. After closing of the leaf gaps, a short ectophloic siphonostele, often still interrupted by branch gaps, is formed in the node (Fig. 8.5c, 8.7e). At this level, the central cavity is replaced by a diaphragm and the vallecular canals are strongly reduced in diameter. As we pass upward above the departure of the leaf traces, the nodal xylem diminishes and becomes separated (Fig. 8.7f, g) with each meristele surrounded by an individual endodermis (Fig. 8.5e). The typical internodal arrangement of vascular bundles with carinal canals and lateral xylem soon reappears (Fig. 8.7h), as well as the internal and external endodermis. Vallecular canals, as well as the central cavity reappear and the leaf sheath is separated (Fig. 8.7h, i). Each internode-node-internode transition shows an alternation in the position of the vascular bundles. The midribs of the leaves, which are fused into a sheath surrounding the base of the next higher internode, are the continuation of the ridges of the lower internode.

Figure 8.4: Transmission (a, b, c) and scanning electron micrographs (d, e, f, g, h) of carinal canals (a, b, c, d), vallecular canals (e), central cavities (f), and xylem (g, h) of *Equisetum ramosissimum*. a. Transverse section of carinal canal and associated parenchyma showing electron dense lining of carinal canal. **b.** Detail of electron dense lining (arrowhead). **c.** Transverse section showing lining of vallecular canal. **d.** Smooth inner surface of carinal canal. **e, f.** Rough inner surface of vallecular canal (e) and central cavity (f). **g.** Multiseriate pitting of nodal xylem elements. **h.** Scleriform pitting of internodal metaxylem elements. Scale bars: a, d, e, f, g, h: 10 μm ; b, c: 2 μm .





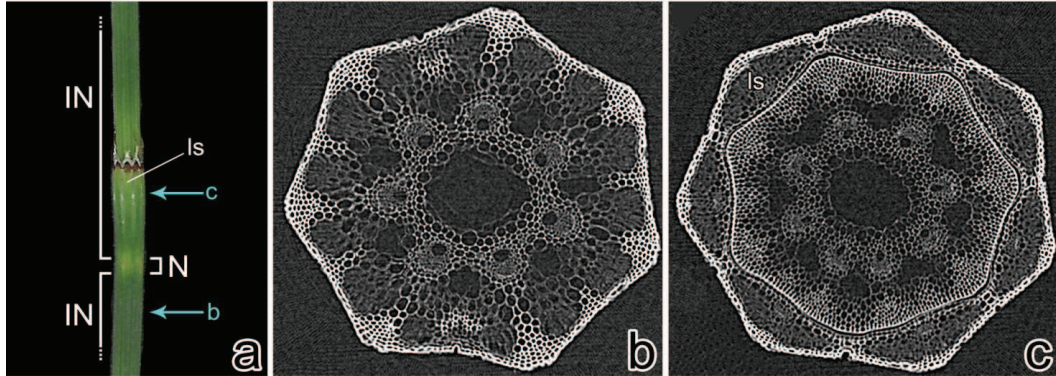


Figure 8.6: Virtual sections through the internode of *E. ramosissimum* based on X-ray CT data. a. Photo showing the position of the node, easily recognizable due to its lighter colour. The basal region of each new internode is surrounded by a leaf sheath (ls). **b, c.** X-ray CT reconstructions showing transverse sections through the internodes, indicated in a. N: node, IN: internode, ls: leaf sheath.

←

Figure 8.5: Longitudinal (a, b, f) and transverse (c, d, e) sections of mature nodes of *Equisetum ramosissimum*. a. Nodal diaphragm (d) interrupting central cavities (cca) of consecutive internodes, carinal canals (cc) connected to nodal xylem (nx). **b.** Detail showing connection of carinal canal (cc) to nodal xylem (nx). **c.** Node showing temporary ectophloic siphlostele interrupted by two branch gaps (arrows), leaf traces (lt) situated outside stele. **d.** Leaf trace (lt) with associated leaf gap (lg) at base of internode-node transition. **e.** Later stage in the node-internode transition with dictyostele consisting of separated meristeles enclosed by an individual endodermis (e). **f.** Nodal region showing bottom of vallecular canal (vc) separated by parenchyma from nodal xylem (nx). Asterisks indicate branch meristems. Scalebars: 100 μ m.

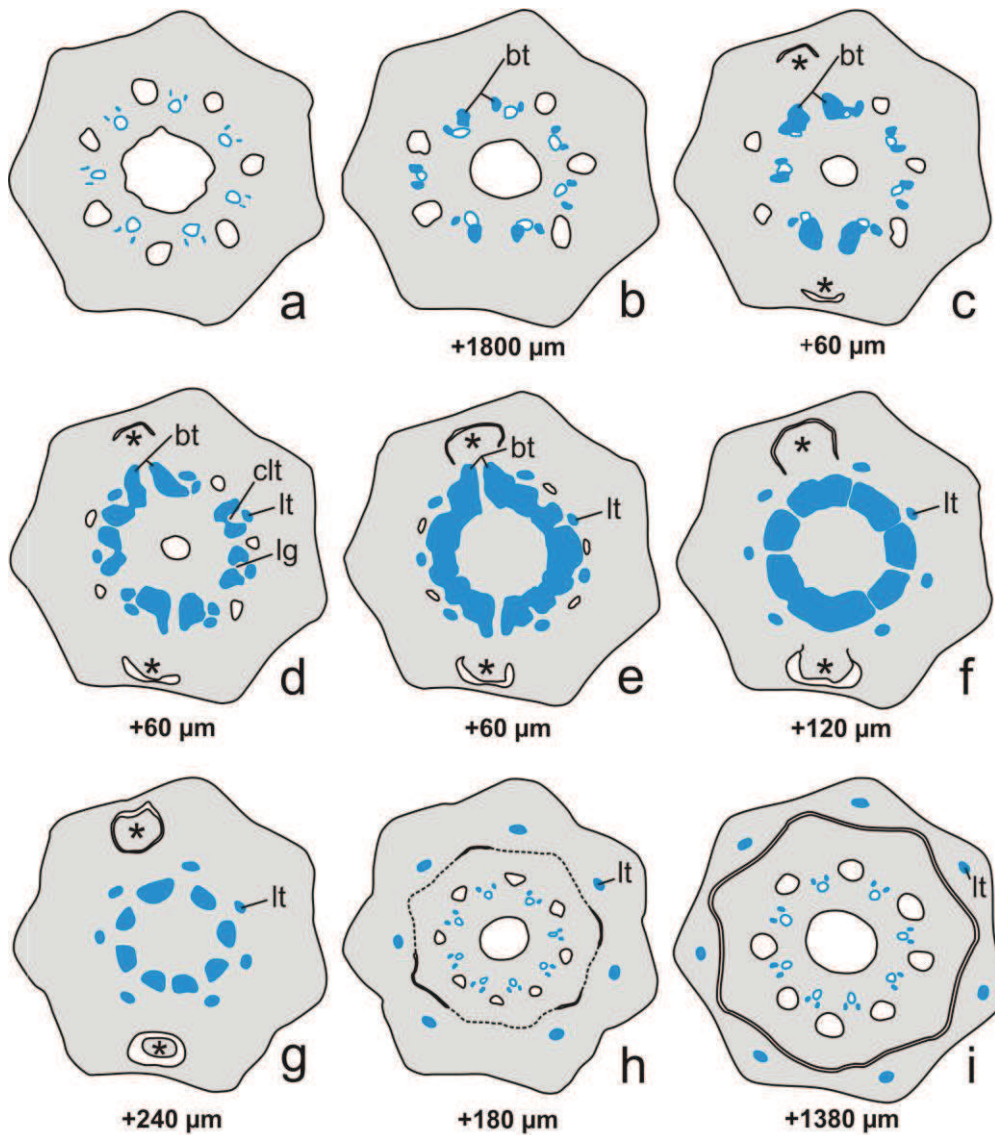


Figure 8.7: Schematic drawings based on X-ray CT slices showing distribution of xylem (blue zones) and carinal canals (blue contours) along internode-node-internode transition. Distance from preceding slice is indicated. **a.** Typical internodal arrangement of vascular bundles with carinal canals and lateral xylem. **b, c.** Extra tracheids extend xylem between lateral metaxylem and carinal canal, outward extension initiating branch traces (bt). **d.** Carinal canals replaced by xylem, leaf traces (lt) branched off and leaving gaps (lg) or becoming closed at this early stage (clg). **e.** Temporary ectophloic sipholostele interrupted by two branch traces (bt). **f, g.** Fragmentation of nodal xylem into meristemes. **h, i.** Separation of leaf sheath and reappearance of typical internodal arrangement of vascular bundles. Branch meristemes indicated with asterisks.

8.4 DISCUSSION

Our study revealed some specific spatial distributions of cell wall epitopes in the stem of *E. ramosissimum*, and provided new data on its general anatomy.

We used a range of monoclonal antibodies to map the distribution of specific cell wall epitopes in *E. ramosissimum* stems with a focus on the carinal canals. Although positive results with labelled antibodies demonstrate the presence of epitopes, negative results do not necessarily imply their absence, as epitopes can be masked by various polysaccharides within cell wall materials even when all cell wall layers are exposed during sectioning (Marcus *et al.*, 2008, 2010). The anti-extensin antibodies LM1 and JIM20 bound strongly to the lining of carinal canals and additional, but very weak, binding to cell wall corners of cortical parenchyma was observed after pectate lyase treatment. To date, extensins have been proposed to be structural cell wall proteins that may play a significant role during growth and development, wound healing, and plant defense reactions (Albersheim *et al.*, 2010). Brownleader *et al.* (2000) have shown that inhibition of tomato hypocotyls growth is mediated, at least partially, by cross-linking of wall extensin. They can be insolubilized in the cell wall through covalent and/or non-covalent cross-linking with themselves and/or other cell wall polymers (Johnson *et al.*, 2003). The presence of JIM5, LM18 and LM19 epitopes and the absence of JIM7 and LM20 epitopes indicated the occurrence of largely de-esterified homogalacturonan in the linings of the carinal canals (Willats *et al.*, 2001). The co-localisation of extensins and highly de-esterified pectins in these linings may suggest some cross-linking between these polymers. Similar co-localization has been reported in cotton fibers (Vaughn and Turley, 1999), in trichomes of mericarps of *Galium aparine* (Bowling *et al.*, 2008), in walls of cellulose-deficient, dichlobenil (DCB)-habituated BY-2 cells (Sabba *et al.*, 1999), and in intercellular pectic protuberances of *Asplenium aethiopicum* (Leroux *et al.*, 2007b). Covalent cross-linking between extensins and pectins has been reported by Qi and Mort (1995). Alternatively, extensins could interfere ionically with the calcium cross-linking of pectic chains, forming weaker gels, which suggests a role for extensins in modulating the pectin-gel network (MacDougall *et al.*, 2001). Extensins can be cross-linked within just a few minutes of wounding, mediated by the release of hydrogen peroxide and catalysed by a wall peroxidase (Cooper and Varner, 1984). Potential cross-linking in the lining of the carinal canals was assessed through a peroxidase activity test, and though we supplied the sample with exogenous H₂O₂, we only observed weak staining of the lining of the carinal canals. Hence we did not obtain strong evidence suggesting peroxidase-mediated cross-linking of extensins. Furthermore, the structural and/or regulatory role of extensins as well as their potential interaction with other polymers in the lining of carinal

canals remain unclear and need to be studied in further detail. We also detected xyloglucan epitopes (LM15/CCRC-M1) in the lining of carinal canals. In this case the epitopes were detected in the lining of carinal canals only after pectate lyase treatment, indicating an intimate association with the pectic homogalacturonan of the lining material. This is distinct to xyloglucan epitope detection in the phloem sieve cells and indicates a distinct disposition of xyloglucan in relation to other cell wall components and unique cell wall architecture in the carinal canal lining. Although we showed that a molecularly-distinct matrix containing de-esterified HG, xyloglucan, extensin and cellulose lines the carinal canals, detailed immunogold localisation studies are required to fully assess the spatial distribution of each of these cell wall components. In *E. arvense*, LM11, recognizing unsubstituted xylan and arabinoxylan, bound to the lining of carinal canals (Sørensen *et al.*, 2008). However, a similar LM11-epitope distribution pattern was not observed in *E. ramosissimum* even following pectate lyase treatment. The binding pattern of the anti-MLG antibody (Biosupplies 400-3) in *E. ramosissimum* was different when compared to that observed by Sørensen *et al.* (2008) in *E. arvense*. Whereas labelling in *E. ramosissimum* was restricted to the strengthening tissue and some cortical cell walls, it was found to strongly bind to the epidermal and cortical parenchyma cell walls in *E. arvense*. Furthermore, Sørensen *et al.* (2008) showed that the MLG-epitopes were distributed throughout the cell walls of the strengthening tissue, while we only detected them in the inner region of these walls. Epitope masking and/or the developmental regulation of MLG incorporation in cell walls may account for the observed differences in epitope distribution.

Electron microscopy showed that the canals are lined with an electron-dense layer, which was absent in the vallecular canals and the central cavity. This electron-dense lining corresponds to the region labelled by the extensin, xyloglucan, cellulose and homogalacturonan antibodies, suggesting the presence of an additional cell wall matrix lining the canals. During light microscopic observations we also noticed that this lining was often thickened in older internodes. A similar moderate thickening was also reported by Bierhorst (1958b) for *E. sylvaticum*. Altogether these observations indicate that this lining is still being modified long after the carinal canals are formed and that additional cell wall material may be deposited in this lining by the cells surrounding these canals. Furthermore, the specialised cell wall matrix lining the carinal canals could be responsible for the smoothness of the carinal canals as observed by scanning electron microscopy.

Since the early detailed investigations on the anatomy of *Equisetum*, it is known that the nodes in *Equisetum* stems are produced in close succession at the shoot apex and

that they become separated by the development of the internodes as a result of intercalary meristematic growth and subsequent elongation (Barrat, 1920; Browne, 1912, 1922). The annular protoxylem elements interconnecting consecutive nodal xylem rings near the shoot apex are stretched and disrupted during the early stages of elongation of the internodes, leading to the formation of the carinal canals (Browne, 1912). The interconnection of carinal canals via the nodal xylem was first reported in *Equisetum* by Sykes (1906), confirmed to occur in *E. sylvaticum* by Bierhorst (1958a), and now also shown to be present in *E. ramosissimum*. Lateral xylem elements only differentiate after the formation of the central, as well as the carinal and vallecular canals, when the internode is fully stretched. The changes occurring during internode-node-internode transition in *E. ramosissimum* are similar to those described for *E. giganteum* (Browne, 1922) and *E. arvense* (Meyer, 1920). Several authors, e.g., Jeffrey (1899), Eames (1909, 1936), Meyer (1920), Smith (1955), Stewart (1983), and Kramer *et al* (1995), stated or accepted that leaf traces in *Equisetum* are not associated with leaf gaps, and therefore considered these to represent microphylls. However, the presence of branching and telome-like leaves in fossil *Hyenia* and *Calamophyton*, the branching open venation pattern in extinct *Sphenophyllum* and *Archaeocalamites* (Lesquereux, 1879; Stewart, 1983; Kaplan, 2001), as well as the presence of leaf gaps in *E. giganteum* (Browne, 1922) and in *E. ramosissimum* (this study) suggest that leaves in horsetails are megaphyllous, which would confirm their separation from the Lycopsidea and their closer alliance to true ferns (Pryer *et al.*, 2001).

The intimate connection of the smooth carinal canals to the nodal xylem indicates that these may represent specialized conducting canals. This was first suggested by Westmaier (1884), and later confirmed by Strasburger (1891), Sykes (1906), Meyer (1920), Buchholz (1921), and Bierhorst (1958b). They studied the water flow in stems of *Equisetum* using dye uptake experiments. Similar experiments have also been undertaken to show water flow in a wide range of monocots (Buchholz, 1921; Dong *et al.*, 1997). Using nuclear magnetic resonance microscopy, Xia *et al.* (1993) demonstrated water flow in the carinal canals of *E. arvense* (misidentified as *E. hyemale*). Their study revealed a relatively low velocity of approximately 40 $\mu\text{m/s}$. This low flow rate most probably resulted from the nodal interruption consisting of very short xylem elements.

8.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The connection of carinal canals from consecutive internodes by nodal xylem rings, suggests that carinal canals function as water-conducting channels. At least during the

elongation of the internodes, when the protoxylem has been disrupted and the metaxylem is not functional yet, the carinal canals are the only structures to transport water. Our immunocytochemical study revealed an extensin-rich cellulose-pectic homogalacturonan-xyloglucan cell wall matrix lining the carinal canals, which most likely corresponds to the electron-dense lining observed with transmission electron microscopy. How this distinct lining relates to the proposed water-conducting function requires further studies.

The distribution of some cell wall epitopes in stems of *E. ramosissimum* was different from that observed in *E. arvense* by Sørensen *et al.* (2008). This may show that cell wall composition might be highly variable among related species. On the other hand, some of this variation might be caused by the fact that cell walls are highly dynamic and, hence constantly remodeled during growth and development, and in response to environmental stress (Lee *et al.*, 2011). Therefore, an increased understanding of structural-functional relationships of polymers (and their interactions) is to be expected when their temporal and spatial occurrence is investigated at cell type level in a taxonomically broad range of plants.

It would also be of interest to investigate the molecular composition of the linings of carinal canals of other *Equisetum* species, or of protoxylem lacunae in other plant taxa such as the intrafascicular canals in the quillwort *Isoetes* (Romeo *et al.*, 2000) and the protoxylem lacunae in the petioles and/or stems of many monocots such as *Nuphar* (Esau, 1965). Comprehensive imaging of cell wall architecture of water-conducting elements in a variety of plant taxa, including bryophytes, lycophytes, ferns, and seed plants has great potential to (1) provide insights into the evolution of vascular tissues, and (2) to allow understanding of how cell wall composition and structure underpins the specialized function of water-transporting elements. For instance, the diversity observed in cell wall composition of water-conducting cells in bryophytes gives new evidence for the multiple origins of water-conducting cells (Ligrone *et al.*, 2002).

A large-scale survey of cell wall structure and composition across the plant kingdom may yield valuable new data which could contribute to our knowledge of cell wall evolution and diversity, as well as to our understanding of how cell wall architecture correlates with specialized properties of specific tissues or tissue types.

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Intercellular Pectic Protuberances in *Asplenium*: new data on their composition and origin.

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Summary

Projections of cell wall material into the intercellular spaces between parenchymatic cells have been observed since the mid 19th century. Histochemical staining suggested that these intercellular protuberances are probably pectic in nature, but uncertainties about their origin, composition and biological function(s) have remained. Using electron and light microscopy, including immunohistochemical methods, we have studied the structure and the presence of some major cell wall macromolecules in the intercellular pectic protuberances (IPPs) of the cortical parenchyma in a specimen of the *Asplenium aethiopicum* complex. IPPs contained pectic homogalacturonan, but no evidence for pectic rhamnogalacturonan-I or xylogalacturonan epitopes was obtained. Arabinogalactan-proteins and xylan were not detected in cell walls, middle lamellae or IPPs of the cortical parenchyma, whereas xyloglucan was only found in its cell walls. Extensin (hydroxyproline-rich glycoproteins) LM1, JIM11 and JIM20 epitopes were detected specifically in IPPs but not in their adjacent cell walls or middle lamellae. We postulate that IPPs do not originate exclusively from the middle lamellae because extensins were only found in IPPs and not in surrounding cell walls, intercellular space linings or middle lamellae, and because IPPs and their adjacent cell walls are discontinuous.

9.1 INTRODUCTION

Aspleniaceae, with over 720 terrestrial, lithophytic or epiphytic species distributed throughout the world, is one of the largest families within the Filicopsida. During a comparative anatomical study of *Asplenium* species we noted projections on cell wall surfaces into intercellular spaces in the cortical parenchyma of the petioles. According to Kisser (1928), De Vriese & Harting were the first to report such excrescences in 1853. Subsequently, similar structures were found in the intercellular spaces of seeds, leaves, stems, and roots of many monocotyledons, dicotyledons, ferns, and fern allies (for a review see Potgieter & van Wyk, 1992). These have been referred to as intercellular wall thickenings (Luerksen, 1873), pectic strands (Carr & Carr, 1975; Carr *et al.*, 1980; Carr *et al.*, 1980b), pectic filaments (Carr & Carr, 1975; Potgieter & van Wyk, 1992), pectic warts (Kisser, 1928; Carlquist, 1956; Carlquist, 1957), scala (Potgieter & van Wyk, 1992), pectic projections (Davies & Lewis, 1981; Veys *et al.*, 1999; Veys *et al.*, 2000; Veys *et al.*, 2002), microprojections (Rolleri, 1993), beads (Jeffree & Yeoman, 1983; Barnett & Weatherhead, 1988), bead-like projections (Miller & Barnett, 1993), papilla-like structures (Suske & Acker, 1989), protuberances (Donaldson & Singh, 1984), intercellular protuberances (Butterfield *et al.*, 1981; Machado *et al.*, 2000), and intercellular pectic protuberances (IPP) (Potgieter & van Wyk, 1992; Machado & Sajo, 1996; Rolleri, 2002; Mengascini, 2002; Prada & Rolleri, 2005; Rolleri & Prada, 2006). Besides *Asplenium*, protuberances in ferns and fern allies have been studied in *Angiopteris* (Carr & Carr, 1975; Rolleri, 2002; Mengascini, 2002), *Pteridium* (Carr & Carr, 1975), *Pteris* (Schenck, 1886), *Blechnum* (Schenck, 1886), *Christensenia* (Rolleri, 1993), *Isoetes* (Prada & Rolleri, 2005), *Equisetum* (Vidal, 1896), *Azolla* (Veys *et al.*, 1999; Veys *et al.*, 2000; Veys *et al.*, 2002) and *Marattia* (Lavalle, 2003).

Intraspecific variability in the occurrence, form and distribution of IPPs has been reported for some Hawaiian Asteraceae (Carlquist, 1957), some southern African Icacaceae (Potgieter & van Wyk, 1992), some *Isoetes* species (Prada & Rolleri, 2005), and for some *Blechnum* species (Rolleri & Prada, 2006). Nevertheless, Potgieter & van Wyk (1992) emphasized the need for more anatomical studies to assess the variability at lower taxonomic levels.

Based on tests using different dyes, such as methylene blue and naphthalene blue, the pectic nature of the protuberances was first postulated by Mangin (1892, 1893). Since then many authors identified pectin as the main constituent of IPPs (Table 9.1). However, 'pectins' constitute a family of polysaccharides rich in galacturonic acid (GalA), and subdivided in three main classes: homogalacturonan (HG), rhamnogalacturonan-I (RGI) and rhamnogalacturonan-II (RGII) (Willats *et al.*, 2006). HG is a linear polymer consisting of 1,4-linked α -D-GalA that can carry varying patterns and densities of

methylesterification. RGI consists of the repeating disaccharide [$\rightarrow$ 4)- α -D-GalA-(1 $\rightarrow$ 2)- α -L-rhamnose-(1 $\rightarrow$)] on which a variety of different glycan chains (principally arabinan and galactan) can be attached to the rhamnose residues. RGII has a backbone of HG rather than RG, with complex side chains attached to the GalA residues (Willats *et al.*, 2006). Using chemical stains, other constituents of the primary cell wall were also detected in IPPs: cellulose in *Picea* (Miller & Barnett, 1993), xyloglucan in *Hymenaea* (Tin   *et al.*, 2000), proteins and callose in *Azolla* (Veys *et al.*, 1999).

The interest in cell walls has increased over the last decennia, and aspects of Pteridophyte and other non-angiosperm plants were studied by Popper & Fry (2003), Popper *et al.* (2004), Matsunaga *et al.* (2004), Popper & Fry (2004), Carafa *et al.* (2005), Popper (2006) and Johnson & Renzaglia (2007). However, only Carafa *et al.* (2006) and Johnson & Renzaglia (2007) used monoclonal antibodies to investigate cell wall composition in Pteridophytes.

Apart from the composition of IPPs, many uncertainties concerning their development remain. Are they formed from materials of the middle lamella during the formation of intercellular spaces, or do they consist of material secreted onto cell walls after the formation of intercellular spaces? The pectic nature of IPPs led most researchers (e.g. Carr & Carr 1975; Carr *et al.* 1980; Potgieter & van Wyk 1992; Tin   *et al.*, 2000) to conclude that IPPs are most probably formed during cell separation. They suggest that when cells are pulled apart during parenchyma expansion, the pectin of the middle lamella becomes stretched and forms strands between adjacent cells. Protuberances then form after the rupture of these strands as parenchyma development proceeds. In contrast to this view, other authors stress the possibility that the pectic filaments observed in ferns (Potgieter & van Wyk 1992; Carr & Carr, 1975) and other plant groups (Machado *et al.*, 2000; Davies & Lewis, 1981 and Butterfield *et al.*, 1981) may be derived from new materials laid down after intercellular space formation. According to Veys *et al.* (2002) protuberances appear both under stress conditions, such as wounding (Davies & Lewis, 1981) and grafting (e.g. Donaldson & Singh, 1983; Miller & Barnett, 1993), and unstressed conditions (e.g. Carlquist, 1956; Potgieter & van Wyk, 1992).

Mostly based on their pectic nature, several possible functions of the intercellular protuberances have been suggested, such as: cell wall hydration, storage, cell adhesion, defense, and apoplastic transport (Potgieter & van Wyk, 1992), but none has been confirmed. Although many authors have described morphological aspects of IPPs, many uncertainties concerning their origin and composition remain. In this paper we describe the intercellular protuberances in *Asplenium*. Using immunohistochemistry and electron microscopy we present new data on their origin and composition.

Table 9.1: Chronological review of studies including test(s) to identify putative chemical component(s) and origin of IPPs. (-) shows authors did not provide data.

Reference	Taxon	Dye(s) or histochemical test(s)	Putative chemical composition	Postulated origin
Mangin (1892, 1893)	<i>Blechnum, Pteris, Equisetum, Vitis, Yucca</i>	Methylene blue, Naphtalene blue, etc.	Pectin	-
Carré & Horne (1927)	<i>Malus</i>	Ruthenium red	Pectin	Secretion
Kisser (1928)	<i>Aconitum, Helleborus, Saxifraga, Daphne, Eriobotrya, Fatsia, Erica, Yucca, Dieffenbachia, Asplenium</i>	Ruthenium red	Pectin	Secretion
Carlquist (1956)	<i>Fitchia, Wyethia</i>	Ruthenium red	Pectin	Secretion
Carlquist (1957)	<i>Argyroxiphium, Wilkesia</i>	Ruthenium red	Pectin	Secretion
Carr & Carr (1975)	<i>Eucalyptus</i>	-	Pectin	Rearranged middle lamella
Carr & Carr (1975)	<i>Angiopteris, Pteridium</i>	-	Pectin	Possibly secretion
Carr <i>et al.</i> (1980)	<i>Vicia, Scilla, Iresin, Helianthus</i>	Pectinase	Pectin	Rearranged middle lamella
Butterfield <i>et al.</i> (1981)	<i>Cocos</i>	Ruthenium red	Pectin	Secretion
Davies & Lewis (1981)	<i>Daucus</i>	Ruthenium red	Pectin	-
Jeffree & Yeoman (1983)	<i>Lycopersicon</i>	Sudan IV Ruthenium red and hydroxylamine/ferric chloride	Neutral lipids Pectin	Secretion
Donaldson & Singh (1984)	<i>Pinus</i>	Toluidine blue	Pectin	-
Potgieter & van Wyk (1992)	<i>Apodytes, Cassinopsis, Pyrenacantha</i>	Ruthenium red and hydroxylamine/ferric chloride	Pectin	Rearranged middle lamella

Miller & Barnett (1993)	<i>Picea</i>	Ruthenium red and hydroxylamine/ferric chloride Nile blue Coomassie Brilliant Blue R Aniline blue Calcofluor	Pectin Fatty acids Protein Callose Cellulose	Secretion
Rolleri (1993)	<i>Christensenia</i>	Ruthenium red	Pectin	-
Machado & Sajo (1996)	<i>Xyris</i>	Ruthenium red	Pectin	-
Veys <i>et al.</i> (1999)	<i>Azolla</i>	Ruthenium red Alcian blue/Toluidine blue Aniline blue, Resorcine blue Coomassie Brilliant Blue R	Pectin Acidic polysaccharides Callose Proteins	Secretion
Machado <i>et al.</i> (2000)	<i>Paepalanthus</i>	Ruthenium red Sudan IV/Sudan black	Pectin Lipids	Secretion
Tiné <i>et al.</i> (2000)	<i>Hymenaea</i>	Iodine PAS reagent	Xyloglucan Carbohydrates	Rearranged middle lamella
Rolleri (2002)	<i>Angiopteris</i>	Ruthenium red	Pectin	-
Veys <i>et al.</i> (2002)	<i>Azolla</i>	Ruthenium red	Pectin	Secretion
Prada & Rolleri (2005)	<i>Isoetes</i>	Coomassie Brilliant Blue R Toluidine blue O	Proteins Pectin	-
Leroux (2005)	<i>Asplenium anisophyllum</i> - <i>complex</i>	<i>Ruthenium red/pectinase</i>	Pectin	Possibly secretion

9.2 MATERIALS AND METHODS

9.2.1 Plant material

Sporophyte material was sampled from plants cultivated at Ghent University Botanical Garden. After examining *Asplenium aethiopicum*, *A. anisophyllum*, *A. balense*, *A. decompositum*, *A. javorkeanum*, *A. nitens*, *A. normale*, *A. onopteris*, *A. protensum*, *A. scolopendrium*, *A. simii*, *A. smedsii*, *A. splendens*, *A. trichomanes* and *A. uhligii*, we selected one specimen (Viane 8539; pers. Herb. R. Viane, GENT) from the *A. aethiopicum* complex for a more detailed analysis. We made sections of both juvenile, uncurling croziers and mature leaves.

9.2.2 Light microscopy

Tissues were embedded using the Technovit 7100 embedding kit (Heraeus Kulzer, Wehrheim, Germany). Small pieces of plant material (root, rhizome, petiole, and lamina), measuring approximately 4 mm at all sides, were excised using razor blades, and fixed in FAA (90% ethanol 50%, 5% acetic acid and 5% commercial formalin). Dehydration was performed using 30%, 50%, 70%, 85% and 94% ethanol. After the last alcohol step, the tissue was infiltrated with hydroxyethylmethacrylate-based resin. Since the samples used in this study are larger than those mentioned in Beeckman & Viane (2000), longer infiltration steps were required. Further treatment was according to Beeckman & Viane (2000), using the one-step embedding method of De Smet *et al.* (2004). Some samples were embedded in custom-made Teflon moulds. Transverse sections of 5 µm were cut with a microtome (Minot 1212, Leitz, Wetzlar, Germany), dried on object glasses, and stained with an aqueous 0.05% [w/v] solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% Na₂B₄O₇. A histochemical test with aqueous 1% [w/v] ruthenium red (Sigma, St Louis, MO, USA) was performed to indicate the presence of pectic material. Handsections were treated with safranin O (Johansen, 1940) and phloroglucinol (Johansen, 1940) to test for lignin, and aniline blue (0.01% in 0.1 M phosphate buffer, pH 9) to demonstrate callose. Micrographs were taken using a Canon EOS D10 mounted on an OLYMPUS BH2 microscope.

9.2.3 Scanning electron microscopy

Material was washed twice with 70% ethanol for 5 min and then placed in a mixture (1:1) of 70% ethanol and DMM (dimethoxymethane) for 20 min. Subsequently, the material was transferred to 100% DMM for 20 min, before it was CO₂ critical point dried using a CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The dried samples

were mounted on aluminium stubs using Leit-C and coated with gold with a SPI-Module™ Sputter Coater (SPI Supplies, West-Chester, PA, USA). Images were obtained on a Jeol JSM-6360 (Jeol, Tokyo) at the Laboratory of Plant Systematics (K.U. Leuven).

9.2.4 Transmission electron microscopy

Petiole cortex tissue was cut into blocks measuring approximately 2 mm at all sides. These were fixed with 2% v/w paraformaldehyde and 2.5% v/v glutaraldehyde in a cacodylate buffer 0.1 M pH 7.4 for 24h at 4°C, washed in the same buffer for 8h, postfixed with 4% osmiumtetroxide and dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100% alcohol/Spurr's resin (1:1) at 4°C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4°C), and transferred to 100% Spurr's resin and left overnight at 4°C. Polymerization was performed at 70°C for 16 h. Seventy-nm-thick sections were made using a Reichert Ultracut S Ultramicrotome. Formvar-coated single slot copper grids were used. Sections were poststained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 10 min in lead citrate stain at 20°C. The grids were examined with a JEM 1010 Jeol electron microscope equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

9.2.5 Immunohistochemistry

The molecular composition of cell walls and IPPs was determined using indirect immunofluorescence with a range of monoclonal antibodies directed against cell wall polysaccharides/glycoproteins. These included anti-HG monoclonal antibodies JIM5, JIM7 and LM7 (Clausen *et al.*, 2003) and PAM1 (Manfield *et al.*, 2005); anti-xylogalacturonan LM8 (Willats *et al.*, 2004); anti-galactan LM5 (Jones *et al.*, 1997), anti-arabinan LM6 (Willats *et al.*, 1998); anti-arabinogalactan-protein antibodies LM2 (Smallwood *et al.*, 1996), JIM4 (Knox *et al.*, 1989), JIM13, JIM14 (Knox *et al.*, 1991) and MAC207 (Pennell *et al.*, 1989); anti-extensin antibodies LM1 (Smallwood *et al.*, 1995), JIM11, JIM12, JIM19 and JIM20 (Smallwood *et al.*, 1994); anti-xylan antibody LM11 (McCartney *et al.*, 2005) and anti-xyloglucan LM15 (Marcus *et al.*, 2008).

Petioles stored in 70% ethanol were sectioned by hand and immediately placed in a fixative of 4% paraformaldehyde in 50 mM PIPES, 5 mM MgSO₄, and 5mM EGTA. Following overnight fixation, sections were washed in PBS and then incubated for 1 h in primary antibody diluted in 5% milk protein in PBS. All rat monoclonal antibodies were used as a 5-fold dilution. Sections were washed in PBS prior to incubation for 1 h in secondary antibody. The secondary antibody was anti-rat IgC coupled to fluorescein isothiocyanate (FITC) (Sigma). Soluble scFV of PAM1 was incubated with sections at 20

$\mu\text{g ml}^{-1}$ followed by, a 100-fold dilution of mouse anti-HIS as secondary antibody. Next, a 50-fold dilution of anti-mouse/FITC was used as tertiary antibody. After washing in PBS, all sections were mounted in anti-fade agent (Citifluor, Agar Scientific) and examined using a microscope equipped with epifluorescence irradiation and DIC optics (Olympus BX-61). Images were captured with a Hamamatsu ORCA285 camera and prepared with Improvision Volocity software. Cellulose was stained with Calcofluor White M2R fluorochrome (fluorescent brightener 28; Sigma; $0.25 \mu\text{g ml}^{-1}$ in dH_2O). In some cases plant material embedded in resin as described above was used for immunohistochemistry.

9.2.6 Peroxidase activity

In-situ peroxidase activity was detected in fresh hand-cut sections of cortical parenchyma following pretreatment in 5 mg ml^{-1} 3,3'-diaminobenzidine (DAB)-HCL, pH 3.8, re-buffered to pH 5.8 immediately before use. Subsequently $1 \text{ mM H}_2\text{O}_2$ was added and incubation was performed at room temperature for 5 minutes.

9.3 RESULTS

We found IPPs in all *Asplenium* species studied. A cross-section of the petiole of a mature *Asplenium* leaf typically shows two vascular bundles (Fig. 9.1A), which fuse towards the rachis. The outermost layer or epidermis is followed internally by a hypodermis consisting of strongly sclerified cells. The largest part of the cortex consists of parenchyma with intercellular spaces (Fig. 9.1A), in which IPPs are visible at magnifications above 200x (Fig. 9.1B). We did not see any regular arrangement of pectic strands connecting adjacent cells.

Sections through superimposed zones of juvenile petioles bearing a crozier show that in the upper zone with undifferentiated metaxylem vessels, the minute intercellular spaces in the parenchyma lack IPPs. In sub-mature tissues IPPs are rare but intercellular space corners are frequently filled with IPP material. Mature petiole bases have large intercellular spaces with numerous IPPs. All mature tissues studied containing intercellular spaces, i.e., the petiolar cortical parenchyma and the laminal mesophyll, possess IPPs. However, relatively few protuberances are present in the mesophyll. Rhizomes have only few and small intercellular spaces that contain very thin filamentous IPPs. Since intercellular spaces are absent in *Asplenium* roots, no cell wall protuberances were observed in this organ.

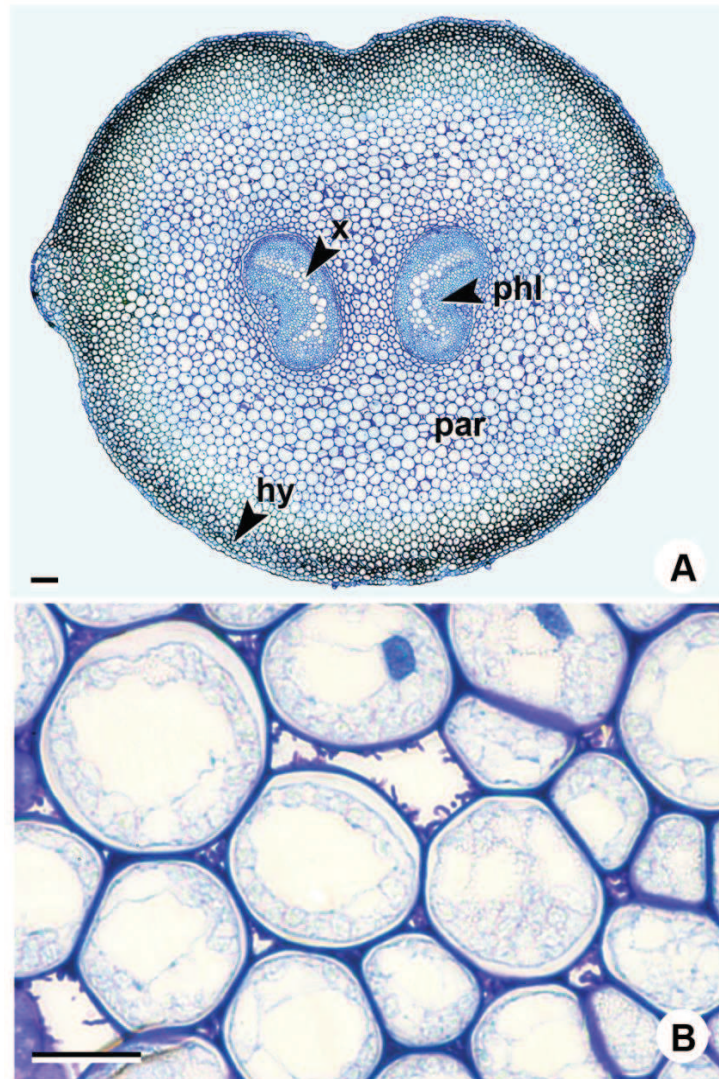


Figure 9.1: Transverse sections of the petiole of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) Light micrograph showing two vascular bundles surrounded by cortical parenchyma. (B) Detail of cortical parenchyma with numerous protuberances lining the intercellular spaces. x: xylem; phl: phloem; par: cortical parenchyma; hy: hypodermis. Bars: a, 125 μm ; b, 30 μm .

Ruthenium red showed a positive reaction with the protuberances, but tests for lignin and for callose were negative. The cellulose-binding fluorophor calcofluor did not label IPPs but clearly bound to neighbouring cell walls (Fig. 9.3C). After performing a peroxidase activity test by adding DAB and H₂O₂ to fresh hand-cut sections, all IPPs in the cortical parenchyma were stained.

Scanning electron micrographs of the cortical parenchyma show protuberances of irregular size and shape, without any regular arrangement or strands connecting adjacent cells (Fig. 9.2A). Warts and nodulated filaments are smooth and appear to be firmly attached to the surface of the cell wall (Fig. 9.2B).

Transmission electron microscopy shows that cell walls are clearly layered and possess simple pits (9.2C, inset). The shapes of IPPs observed with transmission electron microscopy agree with those seen by scanning electron microscopy; their internal structure seems rather homogenous and shows no variation between samples. At higher magnifications an electron-dense fibrillar network is visible within IPPs. The adjacent cell wall shows a more electron-dense outer zone or lining, but there is no continuity between the protuberances and the cell wall (Fig. 9.2D).

The monoclonal antibodies JIM5 and JIM7, which bind to a range of partially methyl-esterified homogalacturonan domains, clearly label the region of the cell wall lining the intercellular spaces between the cortical parenchyma cells. At higher magnifications (Fig 9.3A) the abundance of the JIM5 epitope in the middle lamellae, the intercellular space linings and IPPs is evident. The blockwise de-esterified homogalacturonan epitope recognized by PAM1 binds both to the intercellular space linings and to IPPs (Fig. 9.3D,E). It is of interest that LM5, LM6, LM7, and LM8, monoclonal antibodies that bind to other pectic polymers did not bind to any structure or part of the cortical parenchyma of *Asplenium*. However, the LM6 probe for arabinan bound to all cell walls of the vascular bundle including the endodermis (Fig. 9.3G).

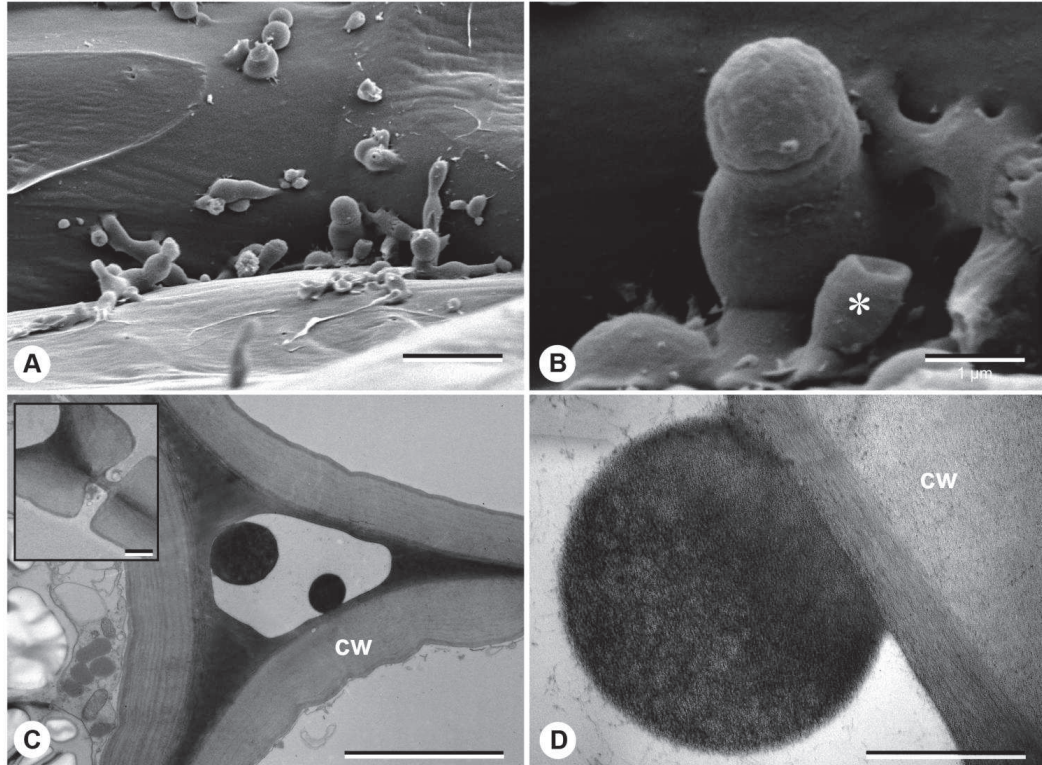


Figure 9.2: IPPs in cortical parenchyma of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) SEM micrograph showing protuberances on cell surfaces in the intercellular space. (B) Detail of a nodulated filament; note some slightly collapsed protuberances due to solvent extraction during processing (asterisk). (C) TEM micrograph of an intercellular space with two wart-like IPPs showing discontinuity in electron-density with the adjacent cell walls (cw); inset: TEM micrograph showing a simple pit. (D) TEM detail of a protuberance showing an internal electron-dense fibrillar network. Bars: A: 5 μm ; B: 1 μm ; C: 4 μm ; inset C: 1 μm ; D: 0.6 μm .

For the non-pectic polymers we found that none of the anti-arabinogalactan-protein antibodies LM2, JIM4, JIM13, JIM14, and MAC207, bound to cortical parenchyma of mature leaves, and that the anti-extensin monoclonal antibodies LM1, JIM11, and JIM20 bound exclusively to IPPs but not to associated cell walls or middle lamellae. The distribution of the LM1 extensin epitope is shown in Figs 9.3H and 9.3I. The monoclonal antibody LM15, recognising the XXXG motif of xyloglucan did not label IPPs, although this antibody did bind to the parenchyma cell walls (Fig. 9.3F). The simple pits, observed by transmission electron microscopy (Fig. 9.2C, inset), were also visualized by this probe. Anti-xylan LM11 bound to the secondary cell walls of tracheids in the vascular tissue but

not to cortical parenchyma cell walls, IPPs or middle lamellae. An overview of our immunohistochemical observations is shown in Table 9.2.

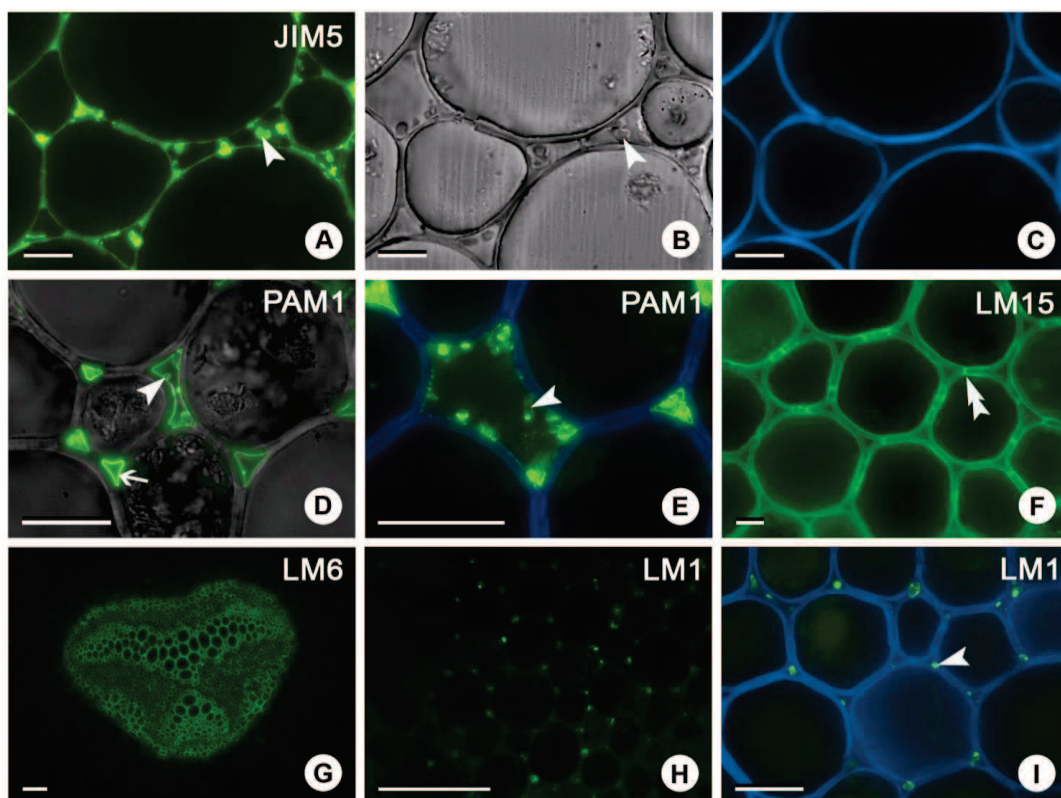


Figure 9.3. Indirect immunofluorescence labelling of cell wall polymers in transverse hand-cut and resin-embedded petiole sections of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) Intercellular spaces with IPPs in the cortical parenchyma (resin-embedded): immunolabelling of HG with JIM5 showing labelled middle lamellae and IPPs. (B) Same section as in (A): differential interference contrast (DIC). (C) Same section as in (A): reaction of cellulose-binding fluorophor calcofluor: note IPPs are not stained. (D) Immunolabelling of unesterified HG with PAM1; bright field/fluorescence micrograph showing binding to IPPs (hand-cut section). (E) Detail of cortical parenchyma labeled with PAM1 and stained with calcofluor. (hand-cut section). (F) Immunolabelling of xyloglucan: note IPPs are not stained (hand-cut section). (G) Immunolabelling of RG-I related (1→5)-α-L-arabinan with LM6: note only the vascular bundle is labelled (hand-cut section). (H) Immunolabelling of extensin with LM1: only IPPs are labelled (hand-cut section). (I) Detail of cortical parenchyma labelled with LM1 and stained with calcofluor (hand-cut section). Arrowheads and arrows indicate IPPs and linings of intercellular spaces, double arrowheads indicate simple pits. Bars: A,B,C,F: 12 μm; D,E,H,I: 30 μm; G: 100 μm.

Table 9.2: Reactions in the cortical parenchyma of a specimen of the *Asplenium aethiopicum* complex (RV 8539) to labelling with monoclonal antibodies directed against cell wall polysaccharides, proteins and glycoproteins. (+): binding; (-): no binding; (?) binding has to be confirmed by immunogold microscopy.

	Antibody	Epitope	Cortex cell walls	intercellular space linings	Middle lamella	IPPs
Pectin						
<i>Homogalacturonan (HG)</i>	JIM5	partially methyl-esterified HG / unesterified HG	-	+	+	+
	JIM7	partially methyl-esterified HG	-	+	+	+
	LM7	partially methyl-esterified HG	-	-	-	-
	PAM1	unesterified HG	-	+	-	+
<i>Rhamnogalacturonan I (RG-I)</i>	LM5	(1→4)-β-galactan	-	-	-	-
	LM6	(1→5)-α-arabinan	-	-	-	-
<i>Xylogalacturonan</i>	LM8		-	-	-	-
Arabinogalactan-protein (AGP)	JIM4	AGP glycan	-	-	-	-
	JIM13	AGP glycan	-	-	-	-
	JIM14	AGP glycan	-	-	-	-
	MAC207	AGP glycan	-	-	-	-
Extensin	LM1	Extensin	-	-	-	+
	JIM11	Extensin	-	-	-	+
	JIM19	Extensin	-	-	-	-
	JIM20	Extensin	-	-	-	+
Xyloglucan	LM15	XXXG motif	+	-	?	-
Xylan	LM11	unsubstituted xylan/arabinoxylan	-	-	-	-

9.4 DISCUSSION

Intercellular protuberances occur in tissues of various ferns and fern allies, gymnosperms, and angiosperms (Potgieter & van Wyk 1992). Because detailed information on protuberances is restricted to relatively few taxa, we emphasize that protuberances with a similar morphology are not necessarily ontogenetically, chemically or anatomically identical.

We confirm Luer's (1875) and Kisser's (1928) observations but not only found big stalked spherical bodies in *A. scolopendrium*, but in all *Asplenium* species studied. IPPs in *Asplenium* are more numerous in the petiole than in the mesophyll. Those in the rhizome resemble the filaments described in the rhizome parenchyma of *Pteridium esculentum* (G.Forst.) Nakai (Carr & Carr, 1975). However, intercellular spaces in the rhizome of *Asplenium* are smaller and protuberances rare.

In contrast to *Cassinopsis ilicifolia* (Potgieter & van Wyk, 1992), transmission electron microscopical observations in our samples showed dissimilarities in electron density between IPPs and their adjacent cell walls and middle lamellae. Remarkably, the primary parenchyma cell walls in this specimen are composed of several layers. This phenomenon has been observed in primary cell walls when they consist of a series of layers in which the orientation of the microfibrils changes by a constant and usually small angle from one layer to the next (Brett & Waldron, 1996).

The protuberances in *Asplenium* show a positive reaction with ruthenium red, supporting their pectic composition. However, results based on reactions with ruthenium red should be interpreted with care because this stain may not be specific enough for pectic compounds (Krishnamurthy, 1999).

The negative tests for lignin and callose seem to indicate the absence of these compounds in IPPs, associated cell walls and middle lamellae. Although cellulose was clearly detected in associated cell walls, it is not found in the protuberances.

As a literature study showed that pectin was believed to be the main substance of intercellular protuberances of many plants, our immunohistochemical tests mainly screened for pectins. The fact that monoclonal antibodies JIM5, JIM7 and PAM1 bind to cell walls and to IPPs indicates the abundance of a mixture of unesterified and methyl-esterified HG. It is of interest that LM7, which binds to a non-blockwise partially methyl-esterified epitope of HG and is restricted to corners of intercellular spaces in angiosperm parenchyma (Willats *et al.*, 2001), did not bind to any material in embedded or hand-cut sections of *Asplenium* cortical parenchyma. Similarly, LM8, an antibody binding to a xylogalacturonan epitope associated with cell detachment in a range of systems (Willats *et al.*, 2004), did not bind to *Asplenium* cortical parenchyma cells, nor did LM5 and LM6,

probes for the possible side chains of pectic RGI: (1→4)-β-galactan and (1→5)-α-arabinan respectively, which have often been correlated with stages of cell and/or tissue development (Ridley *et al.*, 2001).

LM15 xyloglucan-epitopes were detected in cell walls of the cortical parenchyma, but not in IPPs. Consequently, IPPs in *Asplenium* do not have the same chemical nature as the xyloglucan containing protuberances in the storage cell walls in cotyledons of *Hymenaea courbaril* L. (Tin   *et al.*, 2000). We did not detect arabinogalactan-proteins in the cortical parenchyma cell walls or IPPs of our material, though we applied the same extensive range of monoclonal antibodies that was used to demonstrate their presence in angiosperms (Showalter, 2001).

Extensins were detected exclusively in IPPs, indicating that IPP material is distinct from adjacent primary cell walls and middle lamellae. Extensins are abundant constituents of the primary cell walls in mono- and dicotyledons (Smallwood *et al.*, 1995) where they are involved in cross-linking and strengthening processes. As IPPs were stained after performing a peroxidase activity test, extensins in IPPs of *Asplenium* could be cross-linked into IPP structures by this enzyme activity (Brownleader *et al.*, 1993; Everdeen *et al.*, 1988). In addition, extensins are cationic glycoproteins and therefore also have the potential to interact ionically with acidic pectins (Kieliszewski & Lamport, 1994). The distribution pattern of the PAM1 epitope indicates an abundance of unesterified pectin in the IPPs and they therefore may be structurally stabilized by an interaction with extensins.

Few articles report on localisation patterns of peroxidases in fern cell walls. Ros *et al.* (2007) showed that structural motifs characteristic of eudicot S-peroxidases predate the radiation of tracheophytes, since they are found not only in peroxidases from ferns (*Ceratopteris*), lycopods, and basal gymnosperms, but also in peroxidases of mosses and liverworts.

The majority of researchers hypothesize that IPPs are remnants of the middle lamellae, most probably formed during cell separation (e.g. Potgieter & van Wyk 1992; Tin   *et al.*, 2000) when stretched middle lamellae strands snap and form filamentous or wart-like protuberances. However, their presence does not necessarily mean that they are derived from middle lamella components. Substances may be secreted at the site of future intercellular spaces, but prior to their development and expansion, and become incorporated in IPPs during intercellular space formation.

In *Asplenium*, the occasional observation of protuberances stretching completely across an intercellular space, i.e., from the outside of a wall of one parenchyma cell to the outside of another cell wall, could be consistent with the first hypothesis. However,

extensins were only detected in IPPs and not in adjacent cell walls or middle lamellae, indicating a composition distinct from that of the middle lamellae. In addition, transmission electron microscope micrographs showed a clear discontinuity between the protuberances and the cell wall. Overall, these observations strongly suggest that IPPs do not originate exclusively from the middle lamellae.

In summary, this is the first report on the composition of IPPs using monoclonal antibodies directed against cell wall molecules. We identified homogalacturonan and extensin hydroxyproline-rich glycoproteins in IPPs. These results, in combination with transmission electron microscopical observations, suggest that IPPs do not have to originate exclusively from the middle lamellae because additional substances may be secreted during tissue development and become incorporated in IPPs during intercellular space formation. Further biochemical and ontogenetical research on the formation of intercellular spaces is needed to elucidate the composition and possible functions of IPPs in many vascular plants.

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10

Collenchyma in ferns? Perspectives from a histological study of collenchymatous cell walls in *Asplenium rutifolium* (Polypodiales)

10.1 INTRODUCTION

Many plants grow rapidly and the elongating zones are often long and slender. In order to support these regions plants evolved collenchyma, a tissue consisting of elongated cells with thickened primary cell walls, providing both strength and flexibility while maintaining the potential to grow in length (Esau, 1965; Fahn, 1990; Jarvis, 2007). Collenchyma is generally found in stems and leaves of dicots and is rather uncommon in grasses, gymnosperms, and ferns, where sclerenchyma develops at an early stage. Collenchyma occurs as a subepidermal tissue, in a continuous ring or in longitudinal strands, and in association with vascular bundles (Esau, 1965; Fahn, 1990). In leaves, collenchyma is often located on one or both sides of the veins and along the margins of the lamina (Fahn, 1990). Throughout this chapter, I will use the term ‘collenchymatous tissues’ to refer to those tissues comprised of elongated cells with flexible, thickened, cell walls. In contrast to ‘collenchyma’, this term does not necessarily imply that the cell walls are thickened primary cell walls.

Several textbooks (e.g., Esau, 1965; Fahn, 1990) report that ‘collenchyma’ is derived from the Greek word ‘κόλλα’, meaning glue and referring to the thick, glistening appearance of unstained collenchyma cell walls. Notwithstanding the fact that this explanation seems perfectly acceptable, the first use of ‘collenchyma’ in botanical studies was established in the 19th century when Link (1807) used this term to describe the sticky substance on pollen of *Bletia* (Orchidaceae). Two years later, in an anatomical survey of Cactaceae, Schleiden (1839) criticized the use of excessive nomenclature by Link (1837) and stated mockingly that the term ‘collenchyma’ could have more easily been used to

describe elongated subepidermal cells with unevenly thickened cells. Although Schleiden (1839) himself used 'äusere Rindenlage' rather than 'collenchyma' the term seems to have stuck as a way to describe elongated subepidermal cells as it is now widely used.

Harting (1844) perhaps brought 'collenchyma' into common use as he repetitively used 'collenchyma' *sensu* Schleiden in his anatomical survey of annual dicotyledonous Angiosperms. French and English translations of his work soon followed (Giltay, 1882), spreading the new definition or appropriation of 'collenchyma'. That collenchyma was not in common use is perhaps suggested by Van Mohl (1844) who whilst still describing collenchyma tissues as 'jelly-like subepidermal cells' added parenthetically 'the so-called collenchyma cells'. By the end of the 19th century, the term 'collenchyma' was incorporated in most prominent and influential plant anatomy text books and publications (e.g., von Sachs, 1868; de Bary, 1877; Ambronn, 1881; Giltay 1882; van Tieghem, 1886–1888) and was widely used to define elongated cells with unevenly thickened primary cell walls. Ambronn (1881) presented an overview of collenchyma distribution in Dicotyledons and Müller (1890) was the first to recognize three types of collenchymas based on the pattern of thickening: (1) 'angular collenchyma' in which material is deposited in the angles where two or more walls meet, (2) 'lamellar collenchyma' with material mainly deposited on the tangential walls, and (3) 'lacunar collenchyma' with collenchymatous thickenings surrounding intercellular spaces. Duchaigne (1955) added a fourth type, 'annular collenchyma', characterised by evenly thickened cell walls.

Collenchyma walls are generally regarded as being thick primary cell walls. They have been shown to contain high concentrations of pectins and hemicelluloses (Roelofsen, 1959; Jarvis and Apperley, 1990) and often display clear-cut layering, with alternation of layers having a predominantly transverse microfibril orientation and layers having a mainly longitudinal orientation (Beer and Setterfield, 1958; Wardrop, 1969; Chafe, 1970; Pilet and Roland, 1974; Vian *et al.*, 1993; Brett and Waldron, 1996). The layers also vary in composition with layers rich in pectins and poor in cellulose alternating with layers poor in pectins and rich in cellulose (Majumdar and Preston, 1941; Beer and Setterfield, 1958). Fenwick *et al.* (1997) showed that with maturity the mean orientation of microfibrils becomes more longitudinal and the pectins more rigid, with a higher abundance of galacturonans with a low degree of esterification. In more mature organs, collenchyma may develop lignified secondary cell walls (Duchaigne, 1955; Wardrop, 1969). Collenchymatous cell walls have not been extensively studied in terms of their molecular composition. However, probing cell walls with the increasing number of available cell wall-directed monoclonal antibodies and carbohydrate binding modules

(Hervé *et al.*, 2011) is greatly expanding our knowledge regarding cell wall composition of specific tissues. With respect to collenchymatous thickenings Jones *et al.* (1997) showed that a highly specific monoclonal antibody directed against galactan side chains of RG-I specifically bound to the inner cell wall layers of collenchymatous thickenings in tomato petioles. Moreover, the variation of binding patterns of cellulose-directed carbohydrate binding modules showed that cellulose chains in collenchymatous cell walls in celery petioles do not form highly ordered crystalline structures (Blake *et al.*, 2006). Additionally, some xylan-directed probes were shown to bind to distinct regions within collenchymatous cell wall thickenings in tobacco stems (Hervé *et al.*, 2009). These few studies focused, although not focussing on collenchymatous cell walls, indicate that immunocytochemistry has great potential to significantly contribute to our knowledge of the spatial distribution of cell wall polymers within them.

Although collenchyma was one of the first tissues that intrigued botanists with its mechanical role in growth and development (Ambronn, 1881) relatively little is known about its elastic properties (Niklas, 1992). The structural effect of mechanical stimulation on collenchyma tissues of *Datura* and *Apium* (including celery) was investigated by Venning (1949) and Walker (1957, 1960) and their studies showed that mechanical stimulation caused increased cell wall thickening. The mechanical properties of collenchymatous cell walls have further been investigated and demonstrated by Pilet and Roland (1974), Jaccard and Pilet (1975), and Jarvis *et al.* (1984).

Even though, to the best of our knowledge, collenchyma has not been reported to occur in leptosporangiate ferns, we observed collenchymatous tissue in the petioles of 'loxoscapoid' *Asplenium* species, including *A. theciferum*, *A. loxoscapoides* and *A. rutifolium*. These species are mainly distributed in the Afro-montane regions, except for *A. theciferum* which also occurs in mountainous regions of Central and South America. The collenchymatous tissues in these ferns often sclerify, forming a subepidermal continuous layer of sclerenchyma or groups of isolated sclerenchyma bundles. However, in some cases collenchymatous tissues persist throughout the life of the plant and never get to the sclerenchyma stage.

The definition of collenchyma is controversial, partly because it relies on the distinction between primary and secondary cell walls. Primary cell walls are deposited while the cells are still enlarging, whereas secondary cell walls are laid down after elongation has ceased (Carpita & Gibeaut, 1993; Cosgrove, 2005; Albersheim *et al.*, 2010). The cell walls of collenchyma cannot comfortably be defined as either primary or secondary as they thicken during expansive cell growth, and continue with thickening

long after elongation has ceased (Jarvis, 2007). In contrast to mature sclerenchyma which generally consists of dead cells, collenchyma cells stay alive at maturity (Esau, 1965).

Here, we assess the possible occurrence of collenchyma in leptosporangiate ferns through a histological study focusing on the (ultra)structure, cell wall composition and biomechanics of collenchymatous tissues in *A. rutifolium* and by comparison of what already is known about this tissue in angiosperms. We discuss the need for detailed studies and highlight some of the problematic aspects involved in defining collenchyma tissues.

10.2 MATERIAL AND METHODS

10.2.1 Plant material

Asplenium rutifolium was collected in South Africa (RV11506) and kept in the living collection at Ghent Botanical Garden, Belgium.

10.2.2 Vibratome sectioning

6 mm petioles segments were fixed overnight at 4°C in a solution containing 4% paraformaldehyde in PME (50 mM Pipes, 5 mM MgSO₄ and 5 mM EGTA). Following fixation, the samples were thoroughly washed in phosphate-buffered saline (PBS: prepared from a 10x stock solution — 80 g NaCl, 28.6 g Na₂HPO₄·12H₂O and 2 g KH₂PO₄ in 1 L de-ionised H₂O, pH 7.2) and subsequently mounted onto the vibratome stage using superglue. Transverse sections of approximately 50 µm were cut under water with a Microm HM 650 V vibration microtome (Microm International GmbH, Walldorf, Germany) and stored in PBS. Some sections were stained with Delafield's hematoxyline (Johansen, 1940).

10.2.3 Histochemical staining

Phloroglucinol/HCl staining was performed on fresh hand-cut sections using 2% w/v phloroglucinol in 95% v/v ethanol for 5 min, and subsequently mounting in 33% v/v hydrochloric acid. For Maïle staining, fresh hand-cut sections were immersed in 1% (w/v) potassium permanganate for 10 min at room temperature and then washed twice with 3% hydrochloric acid. Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

10.2.4 Resin-embedding and sectioning

Plant material was fixed overnight in FAA (50% v/v ethanol, 5% v/v acetic acid and 5% v/v commercial formalin in distilled water), dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) following Leroux *et al.* (2007) [CHAPTER 2]. Sections of 4 µm were cut with a Microm HM360 microtome (Microm International GmbH, Walldorf, Germany), dried on Vectabond-coated (Vector Laboratories, Burlingame, CA, USA) slides, stained with an aqueous 0.05% w/v solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% w/v Na₂B₄O₇, and mounted in DePeX (Gurr, BDH Laboratory, UK). Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

10.2.5 Transmission electron microscopy

Asplenium rutifolium stems were dissected in order to produce blocks measuring approximately 2 mm³. They were fixed in 4% w/v paraformaldehyde in PME for 2 h at room temperature, washed in the PME buffer and dehydrated in a step-wise gradient of ethanol. The samples were transferred to 100% ethanol/LR White (1:1) at 4°C overnight, brought to 100% ethanol/LR White resin (1:2) for 8 h (4°C), and transferred to 100% LR White and incubated overnight at 4°C. Polymerization was performed at 37°C for 3 days. Seventy-nm-thick sections were made using a Reichert ultracut S Ultramicrotome (Leica, Vienna, Austria). Formvar-coated single slot copper grids were used. Sections were stained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 10 min in lead citrate stain at 20°C. The grids were examined with a JEM-1010 electron microscope (Jeol Ltd, Tokyo, Japan) equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

10.2.6 Scanning electron microscopy

Longitudinal hand-cut root sections were fixed in FAA, dehydrated in a graded ethanol series followed by a graded acetone series, and dried in a critical point dryer (Balzers CPD-030) using CO₂ as a transition fluid. Dried sections were mounted on clean aluminium stubs with double-sided adhesive graphite tabs. Mounted specimens were coated with gold (12–15 nm thick) using a sputter coater (Balzers SPD-050). Sections were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

10.2.7 Immunocytochemistry

For indirect immunofluorescence labelling, hand-cut sections of internodal segments were made. A range of highly specific cell wall-directed probes available from PlantProbes (<http://www.plantprobes.net/>) and Biosupplies (<http://www.biosupplies.com.au/>) was used to evaluate the presence of major cell wall polymers including cellulose (CBM3a; Blake *et al.*, 2006), pectic homogalacturonan (LM19, LM20; Verhertbruggen *et al.*, 2009), (1→5)- α -arabinan (LM6; Willats *et al.*, 1998), (1→4)- β -galactan (LM5; Jones *et al.*, 1997), xyloglucan (LM15; Marcus *et al.*, 2008), xylans (LM10, LM11; McCartney *et al.*, 2005), mannan (LM21, Marcus *et al.*, 2010) and callose (Biosupplies 400-2; Meikle *et al.*, 1991).

The sections were incubated in 5% w/v milk protein in PBS (MP/PBS) for 5 min to block non-specific binding sites. Sections were then incubated with primary rat monoclonal antibodies (LM19, LM20, LM5, LM6, LM10, LM11, LM15, LM21) diluted 1:10 in MP/PBS for at least 1 h. The sections were washed with several changes of PBS prior to incubation with the secondary antibody, anti-rat-IgG linked to fluorescein isothiocyanate (FITC; Sigma) diluted 1:100 in 5% w/v MP/PBS for 1 h. The binding of CBM3a (Blake *et al.*, 2006) was assessed by a three-stage immunolabelling procedure (Hervé *et al.*, 2011). After incubating in MP/PBS containing 40 μ g/ml of CBM3a protein for 1.5 h, sections were washed in PBS at least three times and incubated with a 100-fold dilution of mouse anti-his monoclonal antibody (Sigma) in MP/PBS for 1.5 h. Following washing with PBS, anti-mouse-IgG linked to fluorescein isothiocyanate (FITC; Sigma) was applied for 1.5 h as a 50-fold dilution in MP/PBS. Immunolabelling with the anti-callose antibody 400-2 was performed as described by Meikle *et al.*, 1991. Cellulose was stained with calcofluor white M2R fluorochrome (fluorescent brightener 28, Sigma, 0.25 μ g mL⁻¹ in dH₂O). Subsequently, all sections were washed in PBS buffer three times before mounting in a glycerol-based anti-fade solution (Citifluor AF1, Citifluor Ltd., UK). Recent studies showed that cell wall epitopes can be masked and where appropriate we performed pectate lyase treatments as described by Marcus *et al.* (2008). This treatment removes pectin making other cell wall epitopes accessible to monoclonal antibodies thus enabling labelling. Immunofluorescence was observed with a microscope equipped with epifluorescence irradiation (Olympus BX-61) and images were captured with a Hamamatsu ORCA285 camera and prepared with Improvision Volocity software.

10.2.8 Microtensile tests

Fresh 1 cm long segments were cut from an *A. rutifolium* petiole base. The samples were kept in water for about 1 hour followed by submerging in a 1:1 (v/v) solution of distilled water and polyethylene glycol 2000 (Carl Roth, Karlsruhe, Germany) for three days at 60°C. After incubating for 24 hours in pure polyethylene glycol 2000, samples were embedded and allowed to harden at room temperature. Longitudinal sections (80–100 µm) were made with a rotation microtome (Leica RM2255). Slices with a width of approximately 0.8 mm were cut out and tested in tension using a micromechanical testing device combined with a video extensometry to measure elongation. Wet samples were glued onto foliar frames on both sides resulting in a free sample length of about 2.5 mm and strained at a test velocity of 5 µm/s. Applied forces were recorded with a load cell with a maximum load capacity of 2.5 N. Calculation of mechanical parameters were based on cross section determined for the entire sample.

10.3 RESULTS

A cross section through the petiole base shows an outermost layer or epidermis followed internally by a peripheral zone composed of several layers of collenchymatous cells (Fig. 10.1A). This collenchymatous tissue is interrupted by parenchyma where the respiration lines run along both sides of the petiole. The petiole base displays a typical bilateral symmetric distelic vascular arrangement, in which the xylem of each meristele is arc-shaped (Fig. 10.1A). These meristeles unite higher up in the rachis resulting in a single vascular bundle with an x-shaped xylem strand. Each (meri)stele is enclosed by an endodermal layer with Casparian strips on the radial walls and followed internally by a one to three layered pericycle composed of parenchymatous cells. The xylem is composed of tracheids and xylem parenchyma. Phloem surrounds the xylem on all sides and comprises sieve cells and phloem parenchyma (data not shown). The ground tissue is parenchymatous and contains numerous chloroplasts, especially at the adaxial side. Longitudinal sections show that collenchymatous cells are narrow and elongated with tapered ends (Fig. 10.1B). These cells are in turn subdivided by one to four unthickened transverse cell walls, with a nucleus in each compartment (Fig. 10.1C). The protoplasts appear to be firmly attached to these transverse cell walls (Fig. 10.1C). The collenchymatous tissue in *A. rutifolium* is characterized by uniformly thickened and pitted cell walls which appear translucent in unstained sections (Fig. 10.2A). This semi-transparency results in the green colour of the stipe, caused by the numerous chloroplasts in the ground tissue. Unstained sections also display two distinct layers within the

collenchymatous cell walls: a thin layer which might correspond to the 'primary' cell wall, and a thicker layer which would then constitute the 'secondary' cell wall. The layer that we tend to indicate as the 'primary' cell wall also corresponds to the original cell wall observed in undifferentiated collenchymatous tissue (Fig. 10.2B). However, solely based on the observed layering, it is impossible to state whether or not the collenchymatous thickenings are secondary cell walls. In older fronds, groups of brown-walled sclerified cells are scattered throughout the collenchymatous tissue and correspond with the brown streaks seen on the surface of the petiole. At the petiole base, the collenchymatous tissue is often completely differentiated into sclerified tissue, but this phenomenon is not observed in all plants of this species (data not shown).

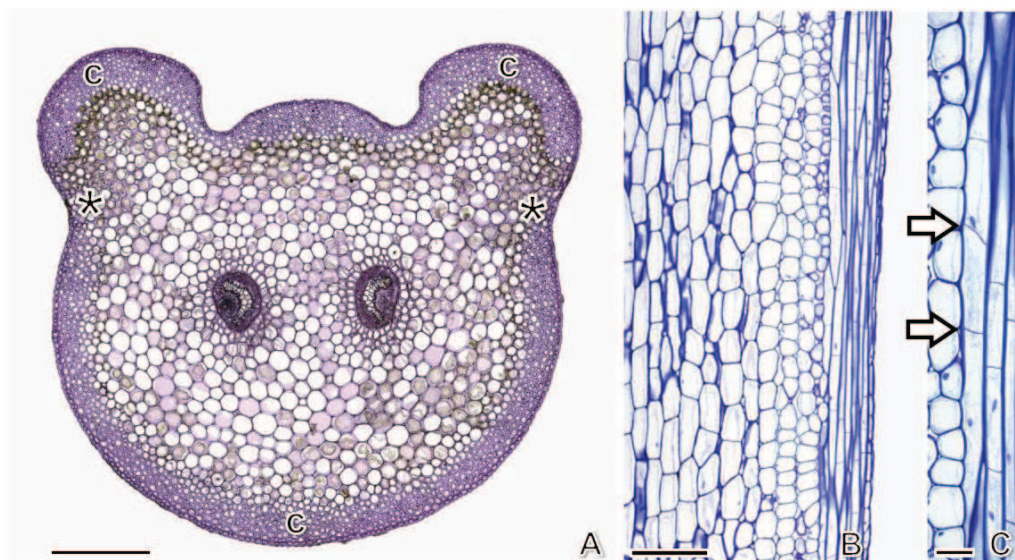


Figure 10.1: Transverse (A) and longitudinal (B, C) sections through the petiole of *A. rutifolium*. A: the subepidermal collenchymatous tissue is interrupted by parenchyma at both respiration lines (asterisks). B: collenchymatous cells are narrow and elongated with tapering ends. C: a higher magnification of B showing unthickened transverse cell walls (arrow), subdividing the collenchymatous cells. Scale bars: A, 1 mm; B, 200 μ m; C, 50 μ m.

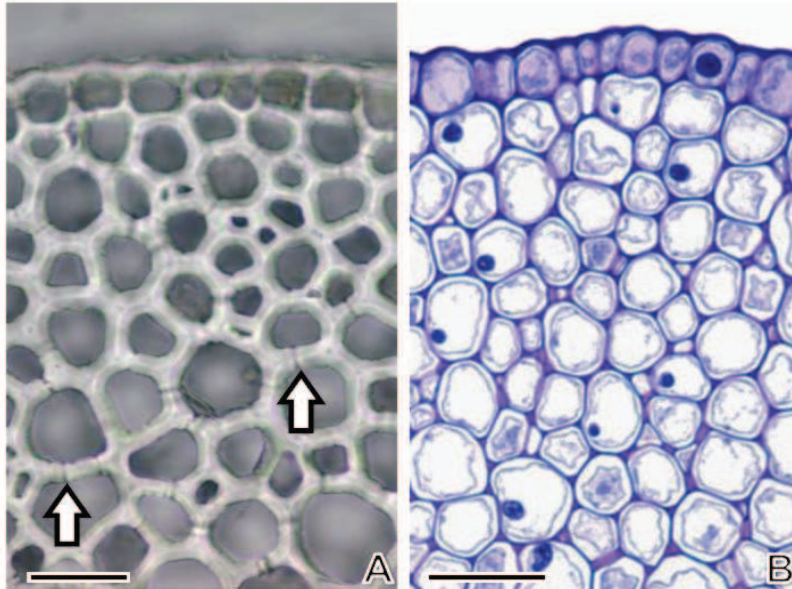


Figure 10.2: Transverse sections of an *A. rutifolium* petiole. **A.** Unstained vibratome section showing the presence of simple pits (arrows) and the tentative 'primary' and 'secondary' cell wall. **B.** Resin-section of undifferentiated collenchyma showing the 'primary' cell walls. Scale bars: 50 μm .

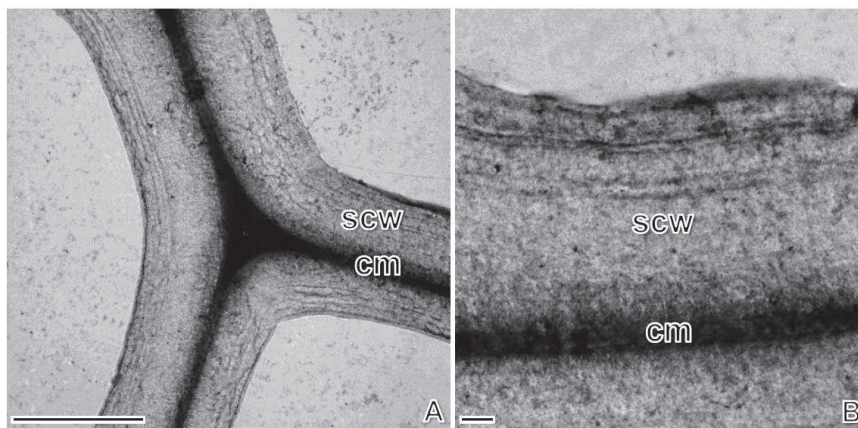


Figure 10.3: Transmission electron micrographs of collenchymatous cell walls of *A. rutifolium* petioles. **A.** Transverse section showing a three-way junction. The electron-dense layer, possibly including the primary cell wall of all cells as well as their intervening middle lamella, is indicated as compound middle lamella (cm). **B.** Detail of the layering of the secondary cell wall (scw). Scale bars: 6 μm .

Transmission electron microscopy did not show similar layering to that observed in unstained sections viewed under light microscopy (Fig. 10.3). However, in many tissues, the middle lamella is often difficult to distinguish from the primary cell wall (Leroux *et al.* 2011 [CHAPTER 5]), especially in cells that develop thick cell walls. Therefore the primary cell walls and their intermediate middle lamellae are often jointly referred to as the compound middle lamella (Esau, 1965). For that reason, and because of the layering observed in our light microscopic study, we tentatively indicated the electron-dense region as the compound middle lamella, and the thick electron-translucent region as the 'secondary' cell wall (Fig. 10.3A, B). The latter showed patterns of stratification (Fig. 10.3B) especially at the inner regions of the wall.

Phloroglucinol/hydrochloric acid and Maûle's reaction only stained protoxylem and metaxylem tracheid cell walls, whereas the collenchymatous cell walls remained unstained (data not shown). To examine the presence of cellulose, callose, hemicelluloses, and pectins, we immunolabelled transverse and longitudinal vibratome sections using a range of monoclonal antibodies and a carbohydrate-binding module. Unless stated otherwise, when antibodies are said to bind to the collenchymatous cell walls, this includes binding to their unthickened transverse cell walls. Sections were also stained with calcofluor white, a dye with a high affinity for (1→4)-β-D-glucans and (1→3)-β-D-glucans (Fig. 10.4A). CBM3a, a carbohydrate-binding molecule which specifically recognizes crystalline cellulose, bound to all tissues, including the collenchymatous cell walls (Fig. 10.3B). We used two monoclonal antibodies which recognise homogalacturonan (HG) either with low, LM19, or with high, LM20, levels of methylesterification. LM19-epitopes were present throughout the collenchymatous thickenings (Fig. 10.3C), whereas the distribution of LM20-epitopes was restricted to the outer cell wall regions and middle lamellae (Fig. 10.4D). LM5, directed against (1→5)-β-galactan, bound only to the inner cell wall layers of collenchymatous cells (Fig. 10.4E) and to the transverse cell walls. The LM6 antibody, recognising (1→5)-α-L-arabinan, did not bind to the collenchymatous cell walls, but was detected in the vascular bundle and epidermal cell walls, and after pectate lyase treatment, also in the cortical parenchyma cell walls (Fig. 10.4F). The xylan antibodies LM10 and LM11 did not bind to the collenchymatous cell walls, and the LM15 antibody, recognizing the XXXG structural motif of xyloglucans, displayed a rather unusual binding pattern being restricted to the filling of three way junctions in the collenchymatous tissue (Fig. 10.4G, H, I).

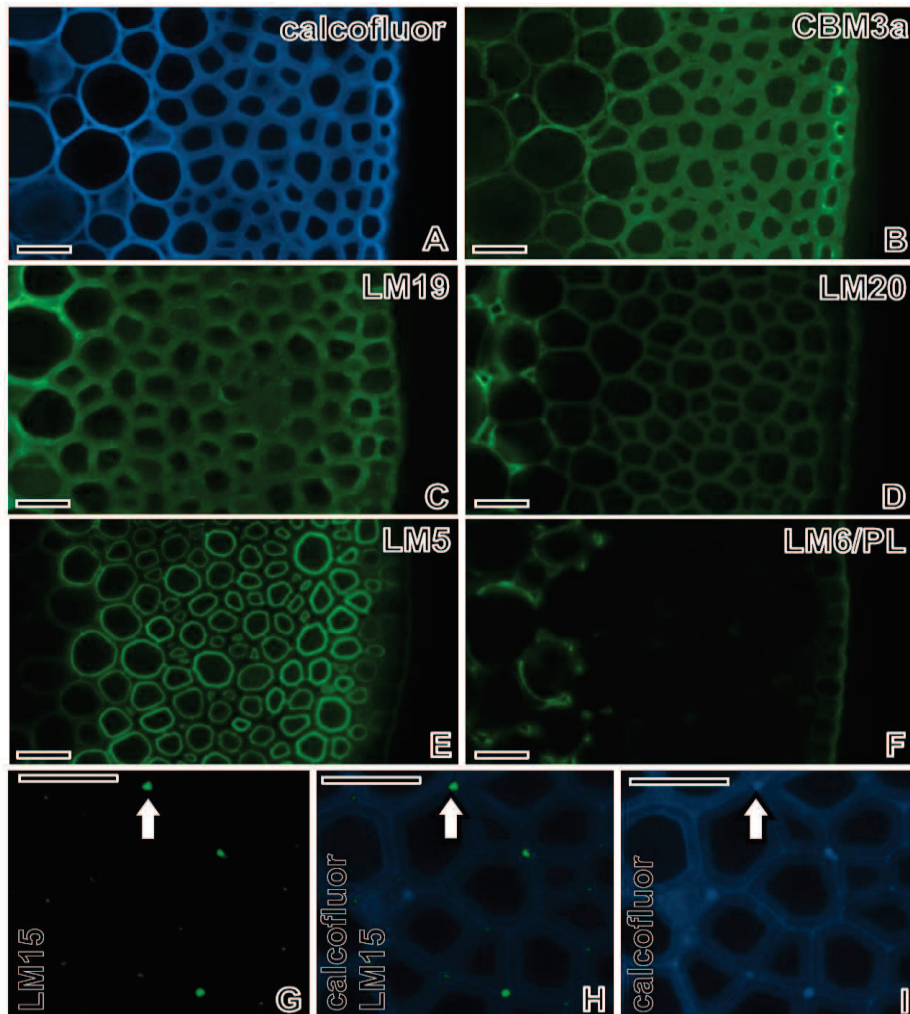


Figure 10.4: Indirect immunofluorescence detection of cell wall epitopes in transverse sections through petioles of *A. rutifolium*. **A:** Calcofluor white fluorescence shows the full extent of cell walls. **B:** Binding of the cellulose directed CBM3a probe to all cell walls, including the collenchymatous cell walls. **C:** LM19, binding to pectic homogalacturonan with low levels of methylesterifications show similar binding patterns as CBM3a. **D:** Binding of LM20, directed against pectic homogalacturonan with high levels of esterification, is restricted to the outer layers of the collenchymatous cell walls, and the middle lamellae. **E:** (1→5)-β-galactan-epitopes, recognized by LM5, were only detected in the inner cell wall layers of the collenchymatous tissue. **F:** (1→5)-α-L-arabinan-epitopes, which are immunolocalised by LM6, only bound to epidermal and cortical parenchyma cell walls after pectate lyase treatment. **G:** Immunodetection of the xyloglucan LM15-epitope in three-way junction of collenchymatous cell walls. **H:** Combined image of calcofluor white staining (blue) and LM15-binding (green). **I:** calcofluor white fluorescence shows the full extent of cell walls. Scale bars: 50 μm.

As reported by Marcus *et al.* (2010), LM21-epitopes can be masked by pectic homogalacturonan. Therefore we treated sections with pectate lyase and this resulted in the unmasking of LM21-epitopes in the outer layers of the collenchymatous cell walls and in the primary cell walls of the cortical parenchyma (Fig. 10.5A, B, C, D). These observations provides further evidence to suggest that the collenchymatous thickenings in *A. rutifolium* are probably secondary cell walls. Interestingly, LM21 did not bind to the transverse cell walls (Fig. 10.5E, F).

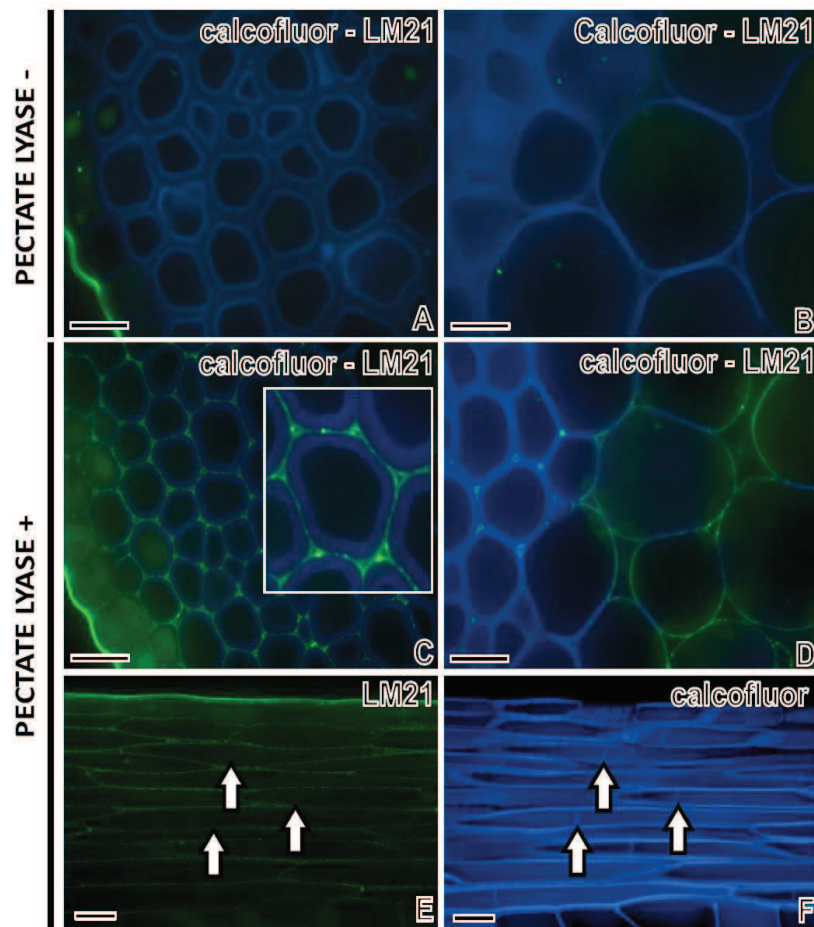


Figure 10.5: Indirect immunofluorescence detection of mannan epitopes in transverse and longitudinal sections through collenchymatous tissues of *A. rutifolium*. **A, B:** No binding of anti-mannan LM21 to collenchymatous (A) and cortical parenchyma (B) cell walls. **C, D:** Pectate lyase pretreatment unmasked LM21 epitopes in the primary cell walls of collenchymatous (C) and cortical parenchyma (D) cell walls. **E, F:** LM21-epitopes are not detected in the transverse cell walls of collenchymatous cell walls. A-D are combined images including calcofluor white staining, showing all cell walls. Scale bars: 50 μ m.

Sclerified collenchymatous cell walls typically showed red autofluorescence (Fig. 10.6C). We did not obtain labelling of these cell walls with any of the antibodies used, except for LM21 which bound to the inner regions of sclerified cell walls (Fig. 10.6A). Additional experiments showed that LM21 labelled inner cell wall regions of the collenchymatous cells prior to phenolic infusion (Fig. 10.6D, E, F). We also used an anti-callose antibody which bound to the transverse cell walls of the cortex parenchyma and collenchymatous tissue (Fig. 10.7A). Scanning electron microscopy of these transverse cell walls showed that they were more densely pitted compared to the longitudinal walls (Fig. 10.7B, C).

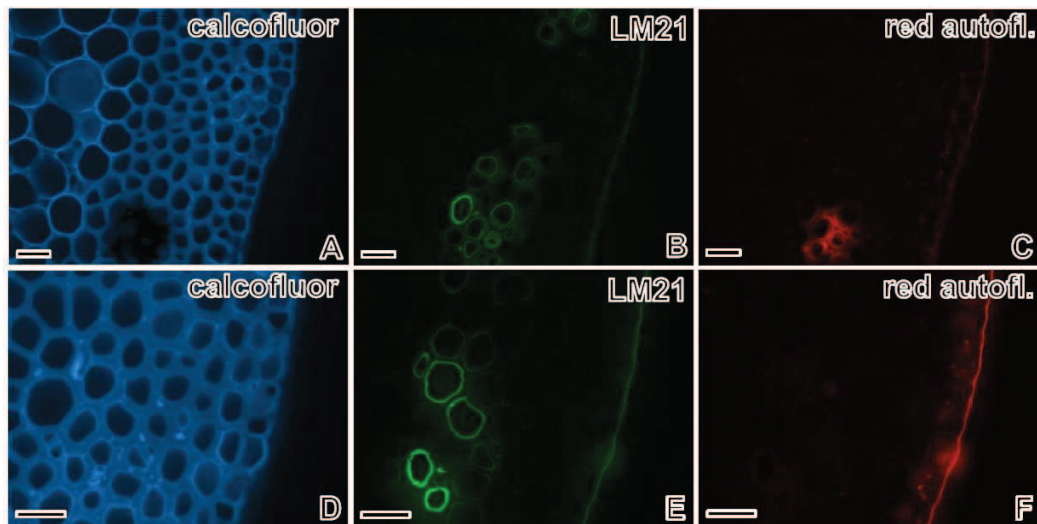


Figure 10.6: Indirect immunofluorescence detection of LM21 mannan epitopes, calcofluor white staining, and red autofluorescence of the same transverse sections through partly sclerified collenchymatous tissues of *A. rutifolium*. Calcofluor white staining shows the full extent of cell walls (B, E) and red autofluorescence indicates that cell walls are infused with phenolic compounds (F). **A–C:** LM21 binds to the inner layer of sclerified collenchymatous cell walls (B). Phenolic infusion of these walls is shown by their red autofluorescence (C). **D–F:** LM21 binds to the inner layer of sclerified collenchymatous cell walls (E). Absence of red autofluorescence (F) shows that these walls are not infused with phenolic compounds. Scale bars: 50 μm .

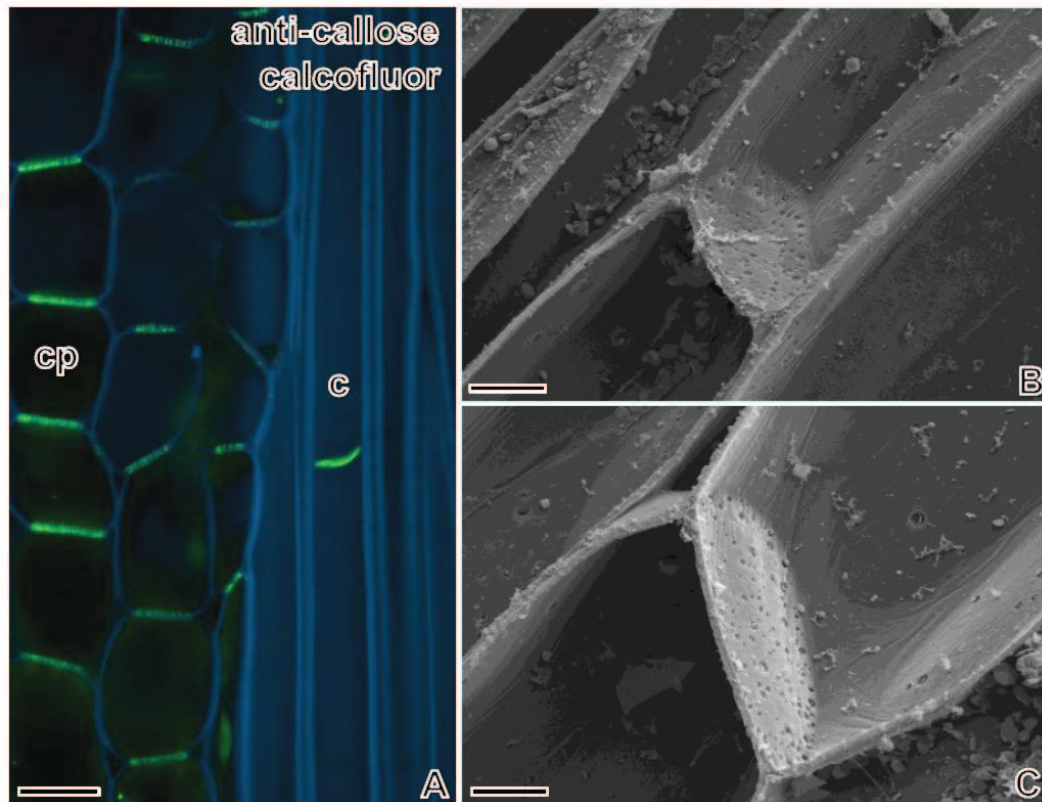


Figure 10.7: Immunodetection of callose and surface view of transverse cell walls of cortical parenchyma and collenchymatous cells in *A. rutifolium*. A. Binding of an anti-callose antibody is restricted to transverse cell walls of both cortical parenchyma (cp) and collenchymatous (c) cells. B, C. Scanning electron micrographs showing the heavily pitted transverse cell walls of collenchymatous (B) and cortical parenchyma (C) cells. Scale bars: A, 50 μm ; B, C, 10 μm .

Microtensile tests were performed on collenchymatous and parenchymatous tissues of *A. rutifolium* and the maximum recorded force (F_{max}), maximum stress (Stress max) and the modulus of elasticity (MOE) are visualised in box-and-whisker-plots (Fig. 10.8). For each parameter we obtained considerably higher values for the collenchymatous tissue than for the parenchymatous tissue. The relationship between the maximum stress and the modulus of elasticity is shown in figure 10.8d. The maximum stress varied in the same direction as the modulus of elasticity: the higher or the lower the stress max, the higher or lower the modulus of elasticity.

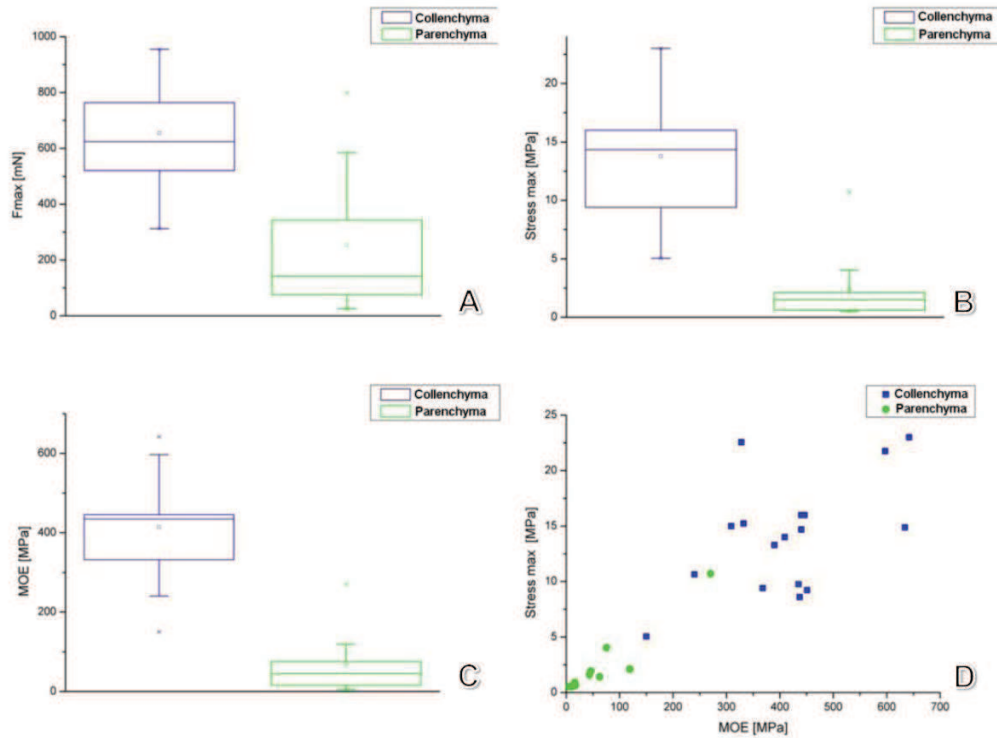


Figure 10.8: Biomechanical parameters of parenchymatous and collenchymatous tissues of *A. rutifolium*. A–C: The maximum recorded force (Fmax), maximum stress (Stress max) and the modulus of elasticity depicted as box-and-whisker plots. 50% of the values inside the box; median: line in the box; mean is indicated with “o”. D: Maximum stress plotted against the modulus of elasticity.

10.4 DISCUSSION

Collenchymatous tissues in *A. rutifolium* petioles consist of elongated cells with tapered ends and uniformly thickened pitted cell walls. Observations of longitudinal sections showed that these collenchymatous cells are subdivided into one or more compartments by unthickened transverse cell walls. By studying unstained collenchymatous cell walls we were able to distinguish two layers which might correspond to ‘primary’ and ‘secondary’ cell walls respectively. We showed that the layer preliminarily designated as the ‘primary’ cell wall matched the original cell wall of the undifferentiated collenchymatous tissue. This layering was, however, not observed by transmission electron microscopy, but it has been shown that the middle lamella is often difficult to

distinguish from the primary cell wall, especially in cells that develop thick cell walls (Esau, 1965).

We did not obtain evidence for the presence of lignins in these walls and the immunocytochemical experiments showed that the collenchymatous thickenings were rich in cellulose and pectic homogalacturonan. The prevalence of the LM19 epitope over the LM20 epitope in the collenchymatous cell walls interestingly suggested the presence of continuous stretches of de-esterified (rather than esterified) HG. However, quite the opposite has been observed in higher plants, where non-esterified HG are particularly abundant in the middle lamellae, and esterified pectins more commonly distributed throughout the primary cell walls (Albersheim *et al.*, 2010). Stretches of non-esterified HG may form gels by calcium cross-linking bridges (Willats *et al.*, 2001a) and the presence of such a gel throughout the wall or in localized regions of the cell wall could affect its physical properties. The variability in methyl-esterification-status of pectic HG consequently must play an important role during cell growth and development, but regulatory and functional aspects of methyl-esterification of HG are not yet fully understood (Verhertbruggen *et al.*, 2009). The LM5 antibody, directed against (1→4)-β-D-galactan, displayed a restricted labelling pattern, binding to the inner region of all collenchymatous cell walls. Similar distribution patterns have been found in collenchymatous cell walls of tomato petioles, hemp stems and leptosporangiate ferns (Jones *et al.* 1997; Blake *et al.* 2008; Leroux *et al.* unpublished results [**Chapter 7**]). It has been postulated by Willats *et al.* (2001b) that the occurrence of RG-I and its structural variants may be related to mechanical properties. For instance, the appearance of galactan, which occurs as side chains on RG-I, has been correlated with an increase in firmness of pea cotyledons (McCartney *et al.*, 2000). McCartney *et al.* (2003), on the other hand, found that the incorporation of the pectic (1→4)-β-D-galactan epitope in cell walls preceded the main phase of cell elongation in *Arabidopsis* roots. Moreover, galactans are enriched in primary cell walls of elongating cells of potato stolons (Bush *et al.* 2001) as well as in elongating carrot suspension cells (Willats *et al.*, 1999). Interestingly, Leroux *et al.* (unpublished results [**Chapter 7**]) found that, in phloem tissues of leptosporangiate ferns, LM5-epitopes were only found in the walls of sieve cells, which, similarly to collenchyma cells, undergo extensive elongation during differentiation. Arend (2008) showed the restricted occurrence of LM5-epitopes to a narrow cell wall area in between the G-layer and the secondary cell wall, strengthening the view that galactan-rich pectins may play an important role in mechanically stressed wood tissue. Pectic galactans are also known to be some of the most flexible cell wall polymers (Ha *et al.*, 1996) and the presence of these polymers decreases the ability of pectin molecules to cross-link and

form a coherent gel network (Hwang and Kokini, 1991). Notwithstanding the fact that structure-function relationships of galactan-rich pectins are still poorly understood, the literature suggests that these polymers might play an important structural and/or regulatory role in mechanically stressed cell walls such as the elongating collenchyma cells in *A. rutifolium*. Arabinans often occur as RG-I side chains and it has been shown that pectic arabinans play a mechanically important role in maintaining the flexibility of guard cell walls (Jones *et al.*, 2003) and are implicated in cell adhesion (Orfila *et al.*, 2001). However, in *Asplenium* LM6 only labelled parenchymatous cell walls of the vascular bundle and epidermal cell walls, but did not bind to the collenchymatous cell walls. It is of interest to note that, in fern cell walls, LM5 and LM6-epitopes might be associated with other polymers than RG-I. For instance, Lee *et al.* (2005) provided evidence that LM6 also binds to arabinogalactan proteins (AGPs) in the moss *Physcomitrella*. One of the major findings is the unmasking of LM21-epitopes in the outer layers of collenchymatous cell walls and cortex parenchyma primary cell walls after removal of pectic HG. This observation suggests yet again that the LM21-labelled layer in collenchymatous cell walls might correspond to its 'primary' cell wall, thus implying that the collenchymatous thickening would then comprise the 'secondary' cell wall. Previously, unmasking of mannan-epitopes in primary cell walls in different plant species has been reported by Marcus *et al.* (2010) indicating that mannans may have a structural role in maintaining primary cell wall integrity. They are also major components of secondary cell walls in gymnosperms and ferns (Whistler and Chen, 1991; Bremner and Wilkie, 1971; Timell, 1962). A comprehensive study of fern cell walls (Leroux *et al.*, unpublished results [Chapter 7]) revealed an association of the mannan LM21-epitopes with sclerified cell walls, further suggesting that mannans are major constituents of the cell walls of early-diverging tracheophytes. Similarly, LM21 did bind to the inner cell wall layer of sclerifying/sclerified collenchymatous cells, indicating that mannans may play an important role in the (early) sclerification process, especially as its incorporation into the cell wall precedes phenolic impregnation. Both xylan-directed antibodies, LM10 and LM11, did not bind to collenchymatous cell walls and removal of pectic HG did not affect their binding pattern indicating that they are not a major component of collenchymatous cell walls in this species. LM15, directed against the XXXG-motif of xyloglucan displayed a very unusual binding pattern as it was restricted to the filling of the tree-way junctions between collenchymatous cells. Similar immunodetection of xyloglucan in intercellular material was reported by Moore *et al.* (1986). The reason for this observed accumulation of xyloglucans — if indeed xyloglucans were immunodetected — is less clear. Altogether, our observations showed an abundance of cellulose, pectic HG, mannan, and galactan in

the collenchymatous cell walls of *A. rutifolium*. Callose was specifically immunodetected in the transverse cell walls of the collenchyma and cortical parenchyma. Scanning electron microscopy showed that these walls were heavily pitted. It is possible that a callose-containing substance is deposited at pit sites. However, callose can be formed by a variety of cells in response to wounding, and may be deposited as plant material is harvested for study (Brett and Waldron, 1996). Detailed studies based on immunogold techniques are needed to investigate the distribution of callose in these cell walls. The microtensile tests performed on collenchymatous and parenchymatous tissue of *A. rutifolium* revealed clear differences between both tissues in terms of their mechanical properties. The MOE of the collenchymatous cell walls was four times higher than that measured for the parenchymatous tissue showing that their resistance to deformation is higher. Based on data of Ambronn (1881), Niklas (1992) mentioned that celery collenchyma has a MOE roughly equal to 22 MPa. We obtained a higher value for the collenchymatous tissue in *A. rutifolium*, which indicates that this tissue is stiffer than celery collenchyma. The MOE of sclerenchyma tissues, on the other hand, is considerably higher. For example, Köhler *et al.* (2000) reported a MOE of 3075 ± 301 MPa for sclerenchyma tissue in *Aristolochia* stems. Comparison of these values shows that the mechanical behavior of the collenchymatous tissues in *A. rutifolium* is more similar to that of celery collenchyma. The higher stiffness of the collenchymatous tissue provides mechanical support to the petiole of *A. rutifolium*. However, the relatively low MOE (compared to that of sclerenchyma) of the collenchymatous tissues indicates that plastic deformations at low stress are possible which is advantageous for this tissue, since it allows growth in length while still being able to increase cell wall thickness. Nevertheless, adequate comparison is currently hampered by the lack of data regarding the biomechanical properties of both collenchymatous and sclerenchymatous cell walls. Ideally, the biomechanical properties of sclerified collenchymatous tissue of *A. rutifolium* should be measured and compared relatively to those of the collenchymatous tissue.

10. 5 CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our preliminary results suggest that collenchymatous cell walls in *A. rutifolium* are most likely non-lignified secondary cell walls which are especially advantageous in young petioles as (1) they enable extensive elongation while maintaining flexibility, and (2) their transparency allows light to enter for photosynthesis in the chloroplasts situated in the ground tissue. Although we clearly showed radial heterogeneity in cell wall composition of collenchymatous thickenings, we do not know

how this observed layering conforms to primary and/or secondary cell walls. If the collenchymatous cell walls in *A. rutifolium* are indeed comprised of both a 'primary' and 'secondary' cell wall, it would be incorrect to refer to this tissue as 'collenchyma', since this tissue has been defined as having thickened primary cell walls. Keeping these things in mind, it is apparent that the definition of collenchyma tissue is more of semantic problem based on the definition of, and distinction between, primary and secondary cell walls. Further studies of the collenchymatous cell walls in *A. rutifolium* are needed, and these should include additional immunolabelling experiments (using more probes directed against cellulose and hemicelluloses), histogenetical studies, determination of lignin content and composition in both collenchymatous and sclerenchymatous tissues via thio-acidolysis, ultrastructural studies, and biomechanical analyses of sclerified collenchymatous tissues.

However, to facilitate full understanding of the composition, structure and biomechanical properties of collenchymatous cell walls, more comparative studies are needed, preferably on a taxonomically diverse range of species. To enable comparison of cell wall composition of collenchymatous tissues of different species, we need to cover all kinds of potential variation in cell wall composition. Therefore it is essential to study the cell wall composition of collenchymatous tissues not only in relation to growth and development, but also in response to environmental stress. Microbiomechanical setups will enable the properties of tissues and individual plant cell walls to be studied, and in conjunction with imaging techniques, they have great potential to reveal the structural and compositional basis of biomechanical properties. As collenchymatous tissues have a clear mechanical function they form an ideal cell wall model for studies on structure-function relationships. Moreover, by comparing biomechanical, compositional and structural aspects of collenchymatous tissues of mechanically stressed and unstressed plants it should be possible to relate the changes in cell wall architecture and composition with those in mechanical properties. In summary, a combinatorial approach is preferred and will provide insights into the structure, composition, function and dynamics of collenchymatous tissues, which will serve as a strong basis for the clarification of its controversial definition.

10.6 ACKNOWLEDGEMENTS

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Part 4

General discussion & future studies

11

General discussion & future studies

11.1 GENERAL DISCUSSION

In this dissertation, various anatomical aspects of ferns have been studied, mainly focusing on cell-wall related anatomical features in Aspleniaceae. As stated in the introduction, fern cell walls have not been studied extensively. However, large-scale surveying of cell walls across the plant kingdom may yield valuable new data that contribute to our knowledge of cell wall evolution and diversity, as well as to our understanding of how cell wall architecture correlates with specialized properties of specific tissues. For that reason, and also because information about fern cell wall architecture is only fragmentary, we performed a comprehensive analysis of fern cell walls using antibody-based approaches.

11.1.1 Aspleniaceae roots: histology and taxonomical significance of anatomical features

If anatomical characters are used as a potential source of systematically useful information it is necessary to understand the range and source of character variation within an individual plant, species or group of related taxa. As will be discussed below, our detailed histological studies did not only provide fundamental insights into plant histology but were also of importance for the detection of potentially taxonomically significant anatomical characters.

In particular, we studied some histological aspects as well as the taxonomical significance of root anatomical characters in Aspleniaceae. Our comparative histogenetical and structural study revealed that the multilayered sclerenchyma sheath originates from two early-separating cell-lineages and that the inner cortex always

remains single layered. This led us to reject the hypothesis of Schneider (1997) that the inner cortex divides periclinally in order to establish a multilayered sclerenchyma sheath. Moreover, we showed that the number of layers of sclereids is highly variable within the same plant or species, and therefore cannot be used as a taxonomically reliable character, as has been proposed by Schneider (1997).

We also studied the distribution of passage zones, which are interruptions in the sclerenchyma sheath. They most likely represent zones offering less resistance to lateral root emergence and/or may serve as a symplastic pathway for water transport between the cortex and stele. Schneider (1997) only found passage zones in the roots of some distantly related species, claiming that the presence of this character is the result of parallel evolution. However, as our screening within Aspleniaceae, using X-ray computed tomography and/or sectioning, revealed the presence of passage zones in the roots of all species studied, we rejected the hypothesis of Schneider (1997). The universal occurrence of passage in roots of Aspleniaceae, but also in many other fern families (Ogura, 1972), most probably relates to their functional role.

We demonstrated the taxonomical significance of helical cell wall thickenings in the outer cortex of Aspleniaceae roots as all taxa possessing such thickenings are grouped in one, strongly supported, monophyletic clade. This led us to reject the hypothesis of parallel evolution of helical cell wall thickenings proposed by Schneider (1997). We postulated that helical cell wall thickenings may stabilise the cortex tissue mechanically, preventing the collapse of tissue during drought periods and allowing a more efficient uptake of water and nutrients during rainfall events.

Despite the fact that some anatomical features are no source of taxonomically valuable information, we were able to confirm some results of Schneider (2004): (1) all species with six inner cortex cells are found in one monophyletic clade, and (2) the mode of cell wall thickening allows discrimination of *Asplenium* from *Hymenasplenium*.

The cell wall composition of the root sclereids had never been studied in detail. Therefore we performed a solid-state carbon-13 nuclear magnetic resonance (NMR) analysis which indicated that hemicellulosic glucomannans dominate by far and that their characteristic yellow-brown colour is most probably caused by oxidation of polyflavonoid tannins. Potential acid-induced transformation of these tannins could then lead to the formation of reddish-brown phlobaphenes, which have been proposed to be responsible for the dark colours of some fern stipes by Ogura (1972). Interestingly, the results also indicated that these hard secondary cell walls are non-lignified. Absence of lignins in the root sclereids was confirmed by acetyl bromide analysis. Unfortunately, there is no published account of the composition of wall preparations solely obtained

from secondary cell walls of leptosporangiate ferns and lignins have only been detected *in situ* in tracheids using the Wiesner staining test (Harris, 2005). In contrast to lignified secondary cell walls, which have been studied extensively, non-lignified secondary cell walls have not received much attention. One of the reasons being that most research groups working on cell walls often either focuss on non-lignified primary or on lignified secondary cell walls (Harris, 2006). However, there is much to be gained by adopting a broader approach to the study of plant cell walls, especially regarding non-lignified secondary cell walls, as these walls show that sclerification does not necessarily imply lignification. More studies are needed to fully explore the presence of lignins in various tissues of leptosporangiate ferns. Are lignins restricted to tracheary cell walls or do they also occur in mechanical tissue?

During our comparative study we observed elongated cell wall-related structures in the cells of the outer root cortex and rhizodermis of many *Asplenium* and *Hymenasplenium* species. These structures have never been reported in Aspleniaceae. Our investigations at the ultrastructural level showed that these structures are formed when cell wall material is deposited on the inner surface of cell wall at the site of microbial penetration, most likely to immobilize these microbes in an attempt to prevent intracellular colonization. Based on the absence of any mycorrhizal structures and the similarity between the CWAs caused by many ascomycetes and the ones we studied, it is likely that ascomycetes are responsible for the CWAs in Aspleniaceae, but it is also possible that they are the result of infection by several different species. Controlled experiments in which (model) ferns (e.g., *Ceratopteris*) are inoculated with specific pathogens, grown in controlled environment cabinets and subsequently sampled for detailed investigations, may shed light on plant-pathogenic interactions in ferns. Moreover, as infection is induced, it will be possible to study the early events during infection. Immunocytochemistry suggested the presence of pectic homogalacturonan, xyloglucan, mannan and cellulose in the cell wall appositions. These epitopes were also detected in the helical cell wall thickenings indicating that a similar regulatory mechanism might be responsible for both cell wall depositions. Despite the fact that similar structures in angiosperm roots have been referred to as 'lignitubers' or 'callosities', we did not detect lignins nor callose, hence our preference to refer to call them 'cell wall appositions', as this term does not put any restrictions concerning their form or composition. We observed that cell walls associated with CWAs were always impregnated with yellowish-brown phenolic compounds. These polymers probably provide high rigidity and indigestibility to host cell walls when they are linked with cell wall carbohydrates such as hemicelluloses and pectins through peroxidase-mediated

cross-linking (Fry, 1986). The infusion of the cell walls with these yellowish-brown components could constitute a first defense reaction to infection. This possibly explains why Schneider (1997) stated that the roots of some *Asplenium* species were characterized by dark brown cortical cell walls.

Altogether, we showed the importance of examining the entire range of a character's variation within and across individuals in relation to ontogeny, environment and the location of the structures in the plant. Anatomical descriptions should only be based on mature specimens as some characters change during maturation. At least some of the observed variation may be related to changes during growth and development, and this is particularly important when considering shape and size. Moreover, variation within an individual plant is to be expected as plants have an open morphogenesis as they grow continuously, and this iterative growth system is made more complex by interacting with the environment. Therefore some characters, being modified during land plant evolution in response to various environmental factors, may be more informative in studies focusing on species-level relationships.

Future studies may focus on functional roles of some structural features such as the passage zones and the helical wall thickenings and should be studied within leptosporangiate ferns. Although many anatomical characters will not necessarily be used to identify species, they are still of importance in phylogenetic studies, especially when they are studied at higher taxonomical levels. First, the conservation of developmental pathways and functional aspects can contribute to the persistence of specific structures in major lineages. Secondly, morphological data are the only set of characters that are observable in both fossil and extant plants. The inclusion of fossils in phylogenetic studies is thus especially significant when studying the tree of life. There is also an increased interest in combining developmental genetics and evolutionary biology to study the evolution of development. In light of the results presented here, it would be of interest to compare the histogeny and developmental genetics of the pericycle, endodermis and lateral root development between representatives of the major lineages (including lycophytes, ferns, gymnosperms and angiosperms). Comparative genome sequences will also present more opportunities to study the evolution of land plant genome structure, and will form a strong basis for evolutionary developmental biology.

11.1.2 Fern cell walls

Our understanding of cell walls of early-diverging land plants (liverworts, mosses, hornworts, lycophytes and pteridophytes) is limited, and there is no clear view of how

they conform to models of cell wall structure that have been developed for model and crop species such as *Arabidopsis* and maize. Plant cell walls are generally classed as extendable primary walls, and secondary walls which are deposited after cell expansion has ceased. In reality, there are no clear boundaries between both cell wall types and they merely represent the extremes of a diversity of specialized cell walls. This diversity is based on the spatial and temporal organisation of cell wall polymers at tissue and tissue-type level in relation to functional requirements, growth and development, and in response to environmental stress. Unfortunately, it is not clear how cell wall polymers and their structural variants are associated with the evolution of cell types, tissues, diverse plant morphologies, or ecological responses.

To investigate the potential tissue-specific and taxonomically significant distributions of cell wall polymers we performed a comprehensive microarray polymer profiling (CoMPP) analysis, complemented with immunocytochemistry, to assess the presence and patterns of occurrence of 34 cell wall epitopes in 76 fern species and some related outliers such as the clubmoss *Lycopodium*. Immuno-based techniques were preferred as they are currently the most efficient technique to provide information concerning the spatial and temporal occurrence of cell wall polysaccharides (epitopes), as information about cell wall heterogeneity is lost when tissues are ground and subjected to biochemical analysis. The results showed that several aspects of cell wall polysaccharides have taxonomically significant distribution patterns and that groups of plants can have specific configurations of polymers within walls of specific cell-types or tissues. For example, the fucosylated xyloglucan epitope CCRC-M1 and the AGP glycan epitope LM2 displayed strong taxonomically linked patterns of occurrence across the ferns, and some pectin, xyloglucan, xylan and mannan-epitopes showed clear associations with specific tissues, cell types or cell wall modifications.

Comprehensive imaging of a variety of cell walls will contribute to our knowledge on cell wall composition with respect to the events of modification during growth, development and in response to environmental changes. To date, the combination of the high throughput advantages of CoMPP-analysis and the *in situ* visualization possibilities of immunolabeling experiments has the greatest potential to significantly extend our understanding of tissue specific cell wall composition and dynamics across the plant kingdom. A future challenge will be to integrate phylogenetic reconstruction, morphological studies and developmental genetic data in order to understand how specific tissues have evolved and how their cell wall architecture and composition have led to their specific functions.

11.1.3 Fern histology: case studies

A specialised cell wall lines the carinal canals in Equisetum ramosissimum

During our comparative study of the occurrence and distribution of specific cell wall epitopes in ferns and lycophytes, we observed a very specific binding of anti-extensin antibodies to the lining of the carinal canals in *Equisetum ramosissimum*, which led us to re-examine the ontogeny, structure, and function of these canals. *Equisetum* is a most unusual genus and, in light of the recent discovery of mixed-linkage glucans in its cell walls (Sørensen *et al.*, 2008; Fry *et al.*, 2008), we believed it was appropriate to focus a detailed study on it. Considerable attention was given to the nodal anatomy, as this has poorly been documented in the recent literature.

We confirmed that carinal canals, connecting superimposed rings of nodal xylem, are formed by the disruption and dissolution of protoxylem elements during elongation of the internodes. Immunocytochemistry revealed the distinct composition of the lining of carinal canals as the LM1 and LM19 extensin epitopes were found to be restricted to these linings. Furthermore, monoclonal antibodies directed against homogalacturonan with a low degree of methyl-esterification, and a cellulose carbohydrate binding module also bound to this lining and xyloglucan epitopes were only detected after enzymatic removal of pectic homogalacturonan. We postulated that carinal canals function as specialised water conducting channels which are especially important during the elongation of the internodes when protoxylem is disrupted and the metaxylem is not yet differentiated. The distinct cell wall composition of the carinal canal linings probably impacts upon its properties and our results show that a clear view on cell wall heterogeneity could contribute significantly to our understanding of how specific configurations of cell wall polymers might result in walls with distinct properties.

Even though we primarily focused on the histology of carinal canals, our survey also revealed the presence of narrow leaf gaps in the node, which might imply that the leaf sheaths in *Equisetum*, consisting of fused uninerval elements, are probably derived from megaphylls. Moreover, we also presented some data concerning the strengthening tissue in *Equisetum*, which has been described either as sclerenchyma (De Bary, 1884; Ogura, 1972; Johnson, 1933; Sørensen *et al.*, 2008) or as collenchyma (Hauke, 1963; Brown, 1976; Kramer 1990). The lack of a consensus definition for this tissue has led to it being given a variety of names including hypodermis (Brown, 1976), hypodermal sterome (Gierlinger, 2008) or strengthening tissue (Spatz *et al.*, 1998; Speck *et al.*, 1998). LM21, an antibody directed against mannan strongly bound to these thickened cell walls. High

quantities of mannans have been found in lower land plants, including *Psilotum* and *Equisetum*-species (Timell, 1964; Popper and Fry, 2004) as well as in the secondary cell walls of gymnosperms (Harris, 2005; Albersheim *et al.*, 2010), suggesting an important structural role in strengthening cell walls. Monoclonal antibodies (LM10 and LM11) raised against xylans and arabinoxylans, polymers which are abundant in sclerenchyma cell walls of many angiosperms (Harris, 2005), were not found to bind to cell walls of the strengthening tissue in *E. ramosissimum*. This suggests the probable absence of xylans from their walls. However, it cannot be excluded that an unusual xylan polymer is present in these cell walls. In addition, we found no evidence for the presence of lignins, suggesting that the cell walls of the strengthening tissue in *Equisetum* are non-lignified.

Intercellular Pectic Protuberances in Asplenium aethiopicum: new data on their composition and origin

Intercellular pectic protuberances (IPPs) are projections of cell wall material into intercellular spaces between parenchyma cells. It is postulated that they are formed as a consequence of the snapping and contraction of stretched filaments of middle lamella material during the formation of intercellular spaces. We studied the (ultra)structure of IPPs using electron and light microscopy, and monoclonal antibodies were employed to examine their composition for the first time. Our results showed the abundance of a mixture of unesterified and methyl-esterified pectic homogalacturonan, and the absence of cellulose and hemicelluloses. Moreover, extensins were detected exclusively in IPPs. This indicated that IPP material is distinct from middle lamella material, suggesting that they do not solely originate from the middle lamellae. The study of the cell wall modifications during the development of three-way junctions as well as the subsequent development of intercellular spaces might provide more insights into the development and possible function(s) of IPPs. It is possible that additional polymers, such as the extensins we localized in the IPPs, might reinforce three-way junctions by providing strong adhesion between cells in order to prevent cell separation. Ultimately, further expansion during tissue growth could open three-way junctions and the pectin-rich sol which once filled the three-way junctions might then form several shapes of IPPs after snapping of stretched material in the developing intercellular spaces.

A preliminary study of collenchymatous tissue of Asplenium rutifolium petioles (Polypodiales)

Although collenchyma has never been reported in petioles of leptosporangiate ferns, 'loxoscapoid' *Asplenium* species, such as *A. theciferum*, *A. loxoscapoides* and *A. rutifolium*, contain subepidermal collenchymatous tissues with uniformly thickened cell walls. We assessed the possible occurrence of 'real' collenchyma tissue in leptosporangiate ferns through a histological study focusing on the (ultra)structure, cell wall composition and biomechanics of collenchymatous tissues in *A. rutifolium*.

Our preliminary results suggested that collenchymatous tissue in *A. rutifolium* consists of elongated cells with non-lignified secondary cell walls which are especially advantageous in young petioles as (1) they permit extensive elongation while maintaining flexibility, and (2) their transparency allows light to enter for photosynthesis in the chloroplasts situated in the ground tissue. Although we clearly showed radial heterogeneity in cell wall composition of these collenchymatous thickenings, there is no clear indication of how, if at all, this layering conforms to primary and/or secondary cell walls. Therefore, if collenchyma is defined as thickened primary cell walls, the recognition of this tissue becomes more of a semantic problem based on the definition of, and distinction between, primary and secondary cell walls. Currently, primary cell walls are viewed as being deposited while the cells are still enlarging, and secondary cell walls as being led down on the primary cell wall when elongation has ceased (Carpita & Gibeaut, 1993; Cosgrove, 2005; Albersheim *et al.*, 2010). However, collenchyma does not fit comfortably into neither of these definitions as its cell walls thicken during cell growth, and continue this process when elongation has ceased. In fact, primary and secondary cell walls merely represent the two extremes of a variety of specialised cell walls. Therefore more work will be needed, at higher resolutions and in conjunction with molecular probes, to explore compositional/structural differences and, hence, the boundaries between these two classes of cell walls. Advance is to be expected through the study of collenchymatous tissues of a taxonomically diverse range of species. A combinatorial approach is preferred and will provide insights into the structure, composition, function and dynamics of collenchymatous tissues, which may serve as a strong basis for the clarification of its controversial definition.

11.1.4 General conclusions

These studies highlight the importance and power of recently developed histological methods to the understanding of plant structure, taxonomy and evolution and demonstrate the scope and future potential of these techniques. In particular, our

comprehensive fern cell wall analysis showed that future studies should focus on specific cell walls and/or tissues, and more precisely on how cell wall components are organized spatially and temporally during growth and development and how cell walls are altered in response to environmental stress. Any efforts made towards the understanding of cell wall design principles and dynamics will also aid prediction of how their architecture and properties might be modified for their improved utilization and through the impact of altered environments. Our observations highlight that (1) more studies are needed to provide a functional, structural and compositional context to explore the boundaries between primary and secondary cell walls, and (2) that ferns and lycophytes should be included in these surveys.

11.2 FUTURE STUDIES

11.2.1 Comparative study of root meristems in land plants

Comparative anatomical studies and fossil evidence combined with phylogenetic interpretation suggests that roots evolved at least twice, once each within the lycophytes and euphyllophytes (Friedman *et al.*, 2004). Unfortunately, detailed developmental studies are still lacking, especially for lycophytes, which show a greater diversity of root apical organisation compared to ferns (von Guttenberg, 1966). Molecular developmental genetics will be very helpful for revealing tissue and organ identity and origin, and should be extended to studies on ferns and lycophytes.

Meristems are complex tissues and the use of X-ray computed tomography could be applied to study their cellular organisation by means of virtual sections enabling comparison of different cutting planes (transverse, longitudinal, oblique) as well as avoiding the labour-intensive serial sectioning and subsequent 3D reconstructions. Figure 11.1 shows virtual sections (based on X-ray CT data) through a (unstained) root tip of *A. elliotii*. Despite the fact that the general structure of the root meristem is mostly evident, discrimination of the individual meristematic cells remains difficult. This is mainly due to the fact that contrast in the resulting X-ray CT images depends on the combination of the density and the chemical composition (atomic number) of the studied matter. High-dense materials (high atomic number or densely packed tissues) absorb X-rays more than low-dense materials (low atomic number or porous tissues) and hence contribute significantly to the contrast of these materials. Primary cell walls of meristematic cells are thin and

largely composed of polymers containing atoms with low atomic numbers (e.g. carbon, oxygen and hydrogen). Therefore, contrasting agents (often applied in electron microscopy) have great potential to enhance the contrast of these cell walls. Some preliminary results are shown in figure 11.2. Conventional contrasting dyes such as uranyl acetate and lead citrate provided excellent results, but these are expensive and toxic. Through experiments with over 30 alternative dyes, we found two cheap and fast staining protocols selectively contrasting either primary (hydroxylamine/ferric chloride) or secondary cell walls (potassium permanganate).

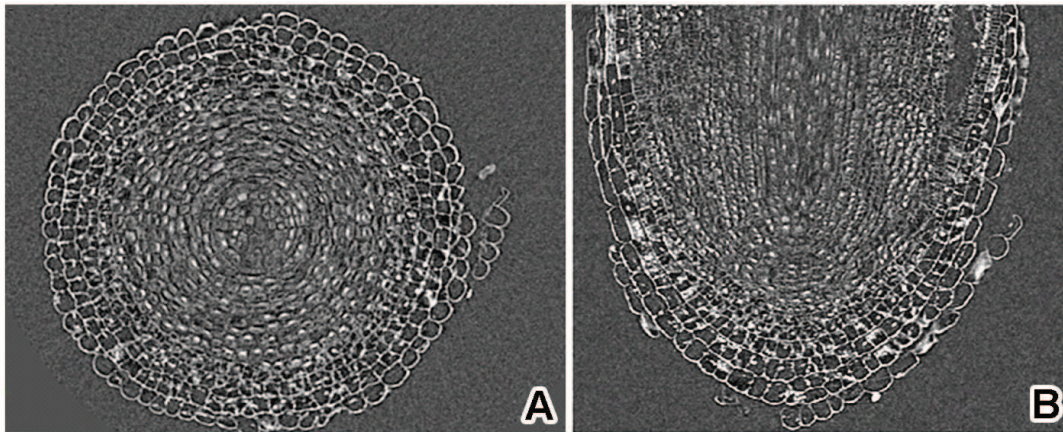


Figure 11.1: Virtual CT-sections of the root apical meristem of *A. elliotii*. A: transverse section, B: longitudinal section (unpublished data, in collaboration with UGCT, UGent).

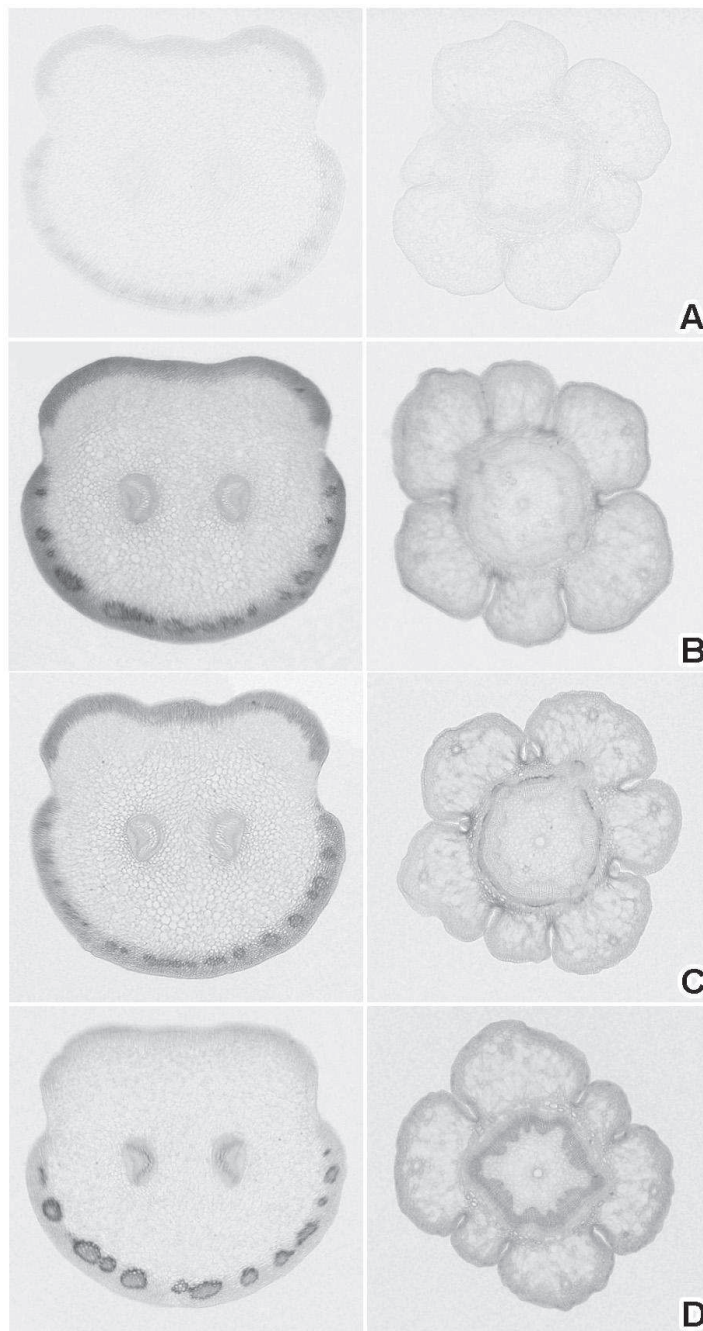


Figure 11.2: The use of contrast-enhancing dyes on transverse vibratome sections of *Asplenium loxoscaphoides* (left) and *Cephalotaxus fortune* (right). A. Unstained control sections. **B.** Sections stained with uranyl acetate and lead citrate. **C.** Sections stained with hydroxylamine hydrochloride and ferric chloride. **D.** Sections stained with potassium permanganate. Note that both hydroxylamine hydrochloride/ferric chloride and potassium permanganate staining significantly enhanced the contrast. The former dye mainly stained primary cell walls, and the latter showed a higher affinity for secondary cell walls. (unpublished data, in collaboration with UGCT, UGent)

11.2.2 Visualisation of cell wall heterogeneity

The increasing availability and use of cell wall monoclonal antibodies and carbohydrate-binding modules enabled the visualisation of previously unknown cell wall heterogeneity and diversity (Lee *et al.*, 2011). Heterogeneity can be observed between walls in different parts of the plant, between walls in a single tissue (Fig. 11.3), and between regions within a single cell wall. For example, light microscopic observation of cortical parenchyma of *Lycopodium squarrosum* showed that this tissue is composed of only one type of parenchymatous cells. However, immunolabelling with the anti-xylan monoclonal antibody LM11, revealed the occurrence of this epitope in cell walls of scattered cortical parenchyma cells (Fig. 11.3).

It will be of interest to know how molecular and structural cell wall heterogeneity relates to both primary and secondary cell walls, as well as to the variety of tissues and tissue-types. As cell wall heterogeneity must impact upon the functions of microdomains within specific walls, its visualisation can significantly contribute to our knowledge of how the molecular architecture of cell walls underpins their specific properties. Moreover, information about cell wall heterogeneity is also of fundamental interest for plant histological studies, as it is mostly by their differing cell walls that cell types or tissues are identified.

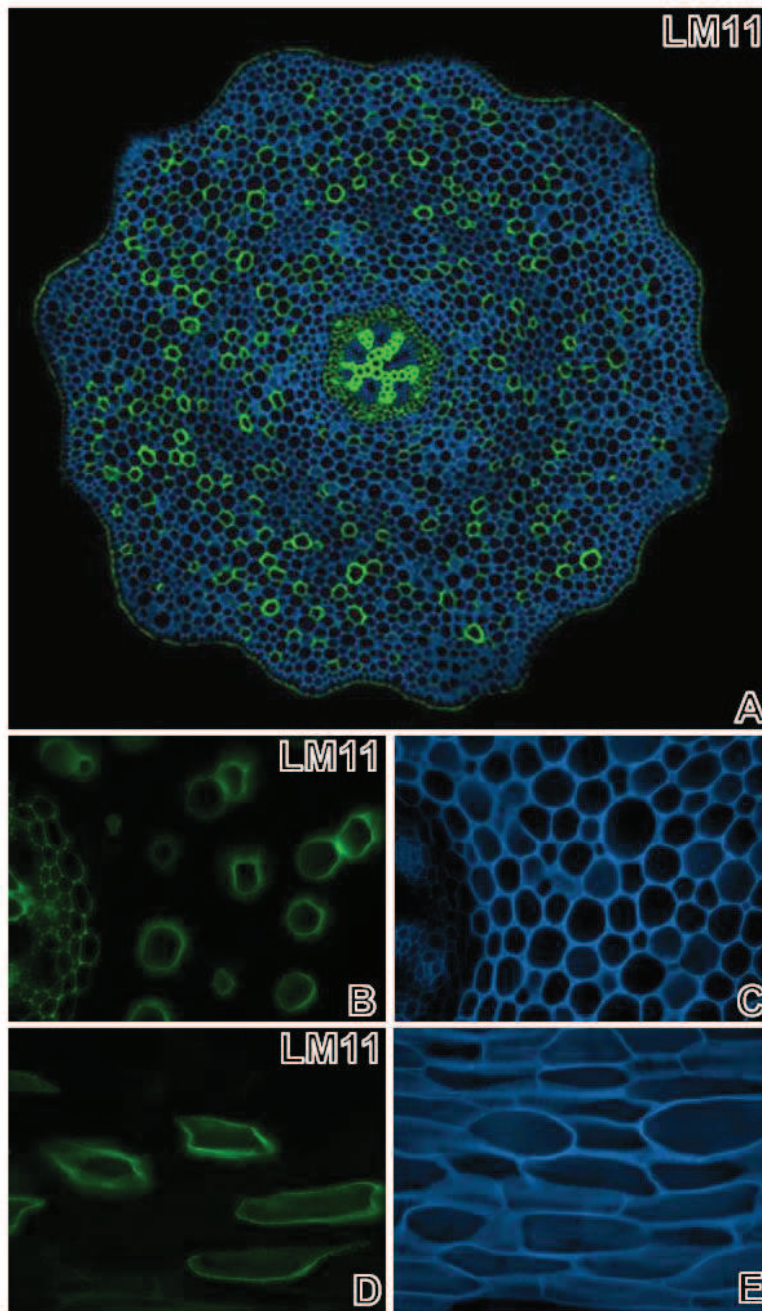


Figure 11.3:
Immunodetection of
LM11 xylan epitopes
in cell walls of
scattered cortical
parenchyma cells in
transverse (A, B, C)
and longitudinal (D,
E) sections through
Lycopodium
squarrosum stems.
Calcofluor White
staining (blue) shows
the full extent of cell
walls.

11.2.3 Evolution and dynamics of collenchymatous cell walls: a structural-functional approach

Plant cell walls are crucial cell components and determine modes of development, specifically cell division, cell enlargement, cell adhesion, and cell differentiation processes. They are dynamic and hence constantly remodeled and reconstructed during growth and development and in response to environmental stress or pathogen attacks. The contemporary view of plant cell walls as sets of highly dynamic and divergent materials leads to many questions including: **(1)** how are cell wall polymers associated with the evolution of cell types, tissues, diverse plant morphologies, and environmental responses, and **(2)** how are polymers associated with tissue-type or cell wall specific properties? Many recent studies, including our comprehensive study of fern cell wall composition [Chapter 7], showed specific associations of cell wall polymers with specific cell types. Therefore, in order to reveal the structural-functional relationships of specific cell wall configurations and the impact of physiological/environmental change, we need to focus at tissue and tissue-type level rather than at organ level. The specialised properties of specific plant tissues or cell types result mainly from the assembly and interaction of polymers within their cell walls. Detailed characterisation of cell walls is thus needed at tissue or tissue-type level to generate understanding into how cell wall architecture results in specialized properties.

Many questions concerning collenchymatous tissues remain: (1) how did collenchymatous tissues evolve and how similar are these tissues among the land plants in which they occur, (2) what is the molecular basis for the mechanical properties of collenchymatous cell walls, and (3) how are these cell walls modified under different environmental conditions?

We aim at studying the development, structure and biomechanical properties of collenchymatous cell walls in a taxonomically diverse range of plants. A comprehensive survey of collenchyma structure in land plants will be carried out and 8–10 species will be selected for a more detailed investigation using a combination of methods including light microscopy, transmission electron microscopy, antibody-based techniques, as well as biomechanical tests. The dynamic properties of cell walls will be assessed by investigating induced modifications in the cell walls of plants grown under different environmental conditions and by studying structural, compositional and biomechanical properties of plants subjected to mechanical perturbation.

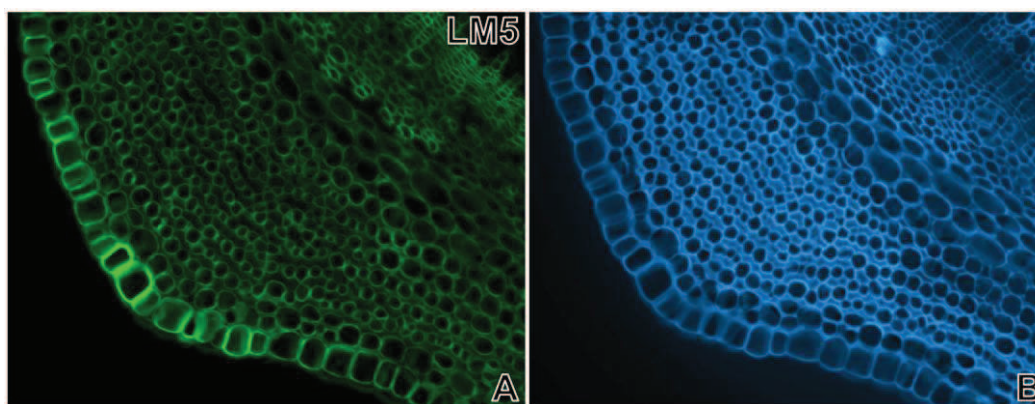


Figure 11.4: Immunofluorescent detection of LM5 arabinan-epitopes in a transverse section of a *Sambucus nigra* stem. A. LM5 binds to the inner cell wall region of collenchymatous thickenings. B. Calcofluor White staining shows the full extent of cell walls.

As discussed earlier, the definition of collenchyma is controversial and needs refinement to include coherence between structure and function and, the proposed research will provide this enabling a clear and contextual description of ‘collenchyma’ to be developed. Research will focus on collenchymatous tissues because: (1) these tissues have a mechanical function and confer flexibility to plant organs, making them the ideal cell wall model for studies on structural-functional relationships, (2) their flexible non-lignified cell walls enable efficient, fast and trouble-free sectioning, and (3) of all major plant tissues, ‘collenchyma’ is not well-defined and efforts to deepen our understanding on collenchymatous cell walls are of fundamental significance.

As well as fundamental insight into the evolution, structure and composition of collenchyma, this project will contribute to our understanding of the organisation of cell wall components and the resultant specific properties induced in the cell wall. Moreover, it will provide insights into how cell walls (and plants) might be impacted in response to climate change. Furthermore, this project coincides with the demand for dynamic cell wall models and the increased interest in cell wall evolution and diversity. Any efforts made towards the understanding of cell wall design principles and dynamics will aid prediction of how cell wall properties may be modified for their improved utilization and through the impact of altered environments. Notwithstanding the fact that we are focussing on collenchymatous cell walls, the results obtained during this project will be of general interest for the plant cell wall community as it will place results of other cell wall comparative studies in a broader context.

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ABSTRACT

FERN HISTOLOGY: A FOCUS ON CELL WALLS AND ASPLENIACEAE

Detailed histological studies of ferns are lacking and yet are of high significance to the understanding of plant evolution and adaptation. During the last decade, many histological techniques have been developed or became more accessible. These include immunolabelling, enabling visualisation of specific components within plant cell walls, which with the development of an increasing array of specific monoclonal antibodies and carbohydrate binding modules, increased capabilities. Further techniques can be applied, sometimes in combination with immunolabelling, to give further details of the (ultra)structure and composition of plant tissues and organs. These include X-ray computed tomography, comprehensive microarray polymer profiling (CoMPP), C13-NMR, transmission electron microscopy, scanning electron microscopy, and laser scanning confocal microscopy.

The cosmopolitan family Aspleniaceae is one of the most species-rich among leptosporangiate ferns with over 720 species of terrestrial, epilithic or epiphytic ferns. Three detailed studies of root anatomical features emphasised the importance of detailed studies in histology: (1) Anatomical studies yielded important information regarding root development. Examination of the structure, composition and ontogeny of the root sclerenchyma sheath showed that this tissue is composed of derivatives of two early separating ontogenetic cell-lineages: a multilayered outer cortex and a single layered inner cortex, and consists of sclereids with non-lignified cell walls rich in mannans and flavonoid tannins. (2) Helical cell wall thickenings occurring in the outer root cortex cells were found to be rich in pectic polysaccharides and hemicelluloses, whereas lignins were absent. (3) A closer examination of cell wall appositions in roots of Aspleniaceae showed that they are defence-related structures made of a non-lignified polysaccharide matrix on which phenolic compounds are deposited creating an impervious barrier which protects the root against infections.

Some of these detailed histological studies did not only provide fundamental insights into plant histology but were also of importance in the elucidation of taxonomically significant anatomical characters. Although anatomy has traditionally proven to be most useful at genus level (or higher) we showed that it can be used to

ABSTRACT

separate subgroups within large genera. Within Aspleniaceae we found three clearly observable taxonomically significant root anatomical characters: (1) the mode of cell wall thickening (uniformly thickened or U-shape thickened), (2) the number of sclereids in the inner cortex (six sclereids or more than six sclereids), and (3) helical cell walls thickenings (presence or absence).

To investigate the potential tissue-specific and taxonomically significant distribution of cell wall polymers in 76 fern species and some related outliers such as the clubmoss *Lycopodium*, we performed a Comprehensive Microarray Polymer Profiling (CoMPP) analysis, complemented by *in situ* immunofluorescence and tissue immunoprinting, using 34 monoclonal antibodies directed against some major cell wall polymers. The results showed taxonomically significant distribution patterns as well as specific associations of cell wall components with cell types, tissues and/or cell wall modifications. Some of the interesting results were studied in more detail.

For instance, we discovered a very specific binding of anti-extensin antibodies to the lining of the carinal canals in *Equisetum ramosissimum* and further examinations showed that these canals have a molecular distinct cell wall lining and function as conducting channels which are especially important during the elongation of the internodes when protoxylem is disrupted and the metaxylem is not yet differentiated.

Intercellular pectic protuberances (IPPs), projections of cell wall material into intercellular spaces between parenchyma cells of the petiole, were studied using electron and light microscopy as well as immunohistochemistry. The results showed that IPPs have a biochemical composition which is different from that of the adjacent cell walls and middle lamellae and suggests that, in contrast to previous conjecture, IPPs do not solely originate from the middle lamellae.

We also undertook a preliminary study of collenchymatous tissues in petioles of *Asplenium rutifolium*, as, to date, 'collenchyma' has not been reported in leptosporangiate ferns. The subepidermal collenchymatous tissues of *Asplenium rutifolium* consist of uniformly thickened, elongated cells with distinctive transverse cell walls. Based on (immuno)histochemistry and transmission electron microscopy, we have postulated that these collenchymatous cell walls are non-lignified secondary cell walls conferring flexibility to petioles. Therefore, if these thickenings are indeed secondary cell walls, this

collenchymatous tissue may not be referred to as collenchyma, as 'real' collenchyma is characterized by thickened primary cell walls.

Altogether these studies provided new data on fern histology and specifically on fern cell walls and generated the first reports of non-lignified secondary cell walls in ferns. The results also highlight the importance and power of recently developed histological techniques to the understanding of plant structure, taxonomy and evolution and demonstrate the scope and future potential of these techniques. Future studies should focus on how cell wall components are organized spatially and temporally during growth and development and how cell walls and morpho-anatomical plant features might be modified in response to environmental stress.

SAMENVATTING

FERN HISTOLOGY: A FOCUS ON CELL WALLS AND ASPLENIACEAE

Op gebied van technische mogelijkheden kende histologisch onderzoek de laatste twee decennia een enorme vooruitgang. Onder andere de beschikbaarheid van monoklonale antilichamen, gericht tegen tal van celwandpolymeren en hun structurele varianten, maakte het mogelijk om de celwandsamenstelling *in situ* te onderzoeken. Dit is van fundamenteel belang aangezien weefsels hoofdzakelijk door hun celwanden worden getypeerd. Andere technieken die ook een steeds grotere beschikbaarheid kennen, zijn elektronenmicroscopie en X-ray computed tomography.

In deze thesis werden verschillende histologische aspecten van varenweefsels en organen bestudeerd en de aandacht ging voornamelijk naar celwand-gerelateerde structuren binnen Aspleniaceae. Aangezien de kennis over de samenstelling van varencelwanden slechts fragmentarisch is, werd met behulp van immunotechnieken een vergelijkende studie van de celwandsamenstelling van varens uitgevoerd om na te gaan in welke mate de distributie van celwandpolymeren taxonomisch significant is en/of geassocieerd is met specifieke weefsels en/of celtypes.

Deze thesis is opgedeeld in vier delen. **DEEL 1** omvat de algemene introductie (**hoofdstuk 1**) waarin diverse aspecten die verband houden met de daaropvolgende hoofdstukken behandeld worden en waarin ook de algemene doelstellingen en de indeling van dit werk worden uiteengezet. Verder worden ook twee methodes voorgesteld die ontwikkeld werden binnen deze doctoraatsstudie. In **hoofdstuk 2** wordt een nieuwe, snelle en goedkope techniek voorgesteld om plantenweefsels en organen volgens een gewenste oriëntatie te kunnen inbedden. **Hoofdstuk 3** bespreekt een aangepaste voorbereidingsmethode om plantenmateriaal te behandelen voor observatie via X-ray computed tomography.

DEEL 2 geeft een overzicht van de histologie en taxonomische bruikbaarheid van celwand-gerelateerde structuren in wortels van vertegenwoordigers binnen Aspleniaceae. De familie van de Aspleniaceae (streepvarenfamilie) is een kosmopolitische familie van meer dan 720 soorten met een opmerkelijke ecologische en morfologische diversiteit. De planten groeien terrestrisch, epilithisch of epifytisch, en

kennen een verspreiding van zeeniveau tot boven 4000 m. Tropische bergregenwouden en zuidelijke gematigde regio's herbergen de grootste diversiteit aan Aspleniaceae, maar sommige soortengroepen wisten zich aan te passen aan een xerische habitat.

In een eerste hoofdstuk (**hoofdstuk 4**) werden de ontogenie, structuur, celwandsamenstelling en taxonomische significantie van de sclerenchymschede onderzocht. De resultaten toonden aan dat deze schede gevormd wordt uit derivaten van twee vroeg-splitsende cellijnen. Vergelijking van de structuur van deze schede binnen Aspleniaceae maakte het mogelijk om de taxonomisch relevantie van twee kenmerken te bevestigen: (1) het type celwandverdikking (uniform of U-vormig), en, (2) het aantal cellen in de binnen cortex (6 cellen of meer dan 6 cellen). De distributie van doorlaatzones werd eveneens onderzocht, en in tegenstelling tot wat voorheen werd gedacht, komen deze in alle soorten voor. Een analyse van de celwandsamenstelling van sclereiden toonde aan dat deze hoofdzakelijk uit glucomannan en flavonoïde fenolen bestaan. Bovendien suggereerden twee onafhankelijke analyses de afwezigheid van lignines.

Hoofdstuk 5 bespreekt enkele histologische aspecten van helicale celwandverdikkingen in de wortelcortex. Deze verdikkingen zijn niet-gelignificeerde celwanden die rijk zijn aan cellulose, hemicelluloses en pectines. Alle soorten met deze celwandverdikkingen in de cortex vormen samen een monofyletische groep bestaande uit voornamelijk epifyten. Wij suggereren dat deze structuren het cortexparenchym beschermen tegen inklappen tijdens droogte, en dus een belangrijk voordeel zouden kunnen vormen bij epifyten.

Celwandapposities zijn verdedigings-gerelateerde structuren die gevormd worden in de rhizodermis en buitenste lagen van de wortelcortex wanneer celwandmateriaal afgezet wordt op plaatsten waar micro-organismen de cel proberen binnen te dringen. In **hoofdstuk 6** werd de samenstelling van dergelijke celwandapposities onderzocht en werd met behulp van transmissie elektronenmicroscopie bevestigd dat micro-organismen - vermoedelijk pathogene fungi - volledig ingebed zijn in celwandmateriaal.

DEEL 3 bundelt alle hoofdstukken waarin de celwandsamenstelling op verschillende niveaus (weefsel- en celwand specifiek) werd onderzocht. Celwanden zijn een divers en onmisbaar onderdeel van plantencellen. Naast hun andere functies liggen ze aan de basis van het behoud van de celvorm en dragen ze bij tot de structurele integriteit en morfologie van de gehele plant. Terwijl begrijpelijk de meeste aandacht gaat naar celwanden van economisch belangrijke bloemplanten zijn slechts een beperkt aantal publicaties beschikbaar over de moleculaire structuur van celwanden van

wolfsklauwachtigen en varens. Onderzoek op deze landplanten krijgt steeds meer aandacht omdat de fundamentele kennis van de spatio-temporele distributie van celwandmoleculen in deze “primitievere” organismen een niet te onderschatten meerwaarde kan bieden om het belang en de functie van deze moleculen in (economisch belangrijke) bloemplanten te begrijpen. Evenmin is bekend in welke mate celwandpolymeren en hun structurele varianten geassocieerd zijn met de evolutie van celtypes, weefsels en organen.

In een eerste studie werd de celwandsamenstelling van 76 varens en 4 wolfsklauwen onderzocht met behulp van antilichaam-gebaseerde technieken (Comprehensive Microarray Polymer Profiling-analyse, immunokleuringen op vibratoomcoupes en weefselprints). De resultaten toonden aan dat (1) de distributiepatronen van bepaalde celwandepitopen taxonomisch relevant zijn, en (2) dat sommige celwandepitopen geassocieerd voorkomen met specifieke weefsels, celtypes en/of celwandmodificaties.

De ontdekking van mixed-linkage glucan in de celwanden van *Equisetum* zorgde voor een toenemende interesse in dit genus. Daarom werd de *in situ* celwandsamenstelling, en in het bijzonder die van de celwandaflijning van de carinale kanalen, in detail onderzocht (**hoofdstuk 8**). Immunokleuringen op coupes doorheen de stengel van *Equisetum ramosissimum* toonden een afwijkende celwandsamenstelling van de aflijning van de carinale kanalen aan, bestaande uit pectines, extensines, cellulose en xyloglucan. Een gedetailleerde anatomische studie van de overgang van knoopstuk naar tussenknoopstuk toonde verder aan dat carinale kanalen van de tussenknoopstukken via xyleemringen in de knoopstukken met elkaar verbonden zijn. Deze bevindingen suggereren dat (1) carinale kanalen een gespecialiseerde coating hebben, en (2) waarschijnlijk functioneren als transport kanalen. Deze transportfunctie is vooral belangrijk tijdens de elongatiefase van de tussenknoopstukken, aangezien xyleem elementen op dat ogenblik nog niet gedifferentieerd zijn.

Intercellulaire Pectine Protuberansen (IPP's) zijn celwand-geassocieerde wrat- of filamentvormige structuren in intercellulaire ruimtes van parenchymatische weefsels. Hoogstwaarschijnlijk worden deze structuren gevormd tijdens de ontwikkeling van intercellulaire ruimten wanneer pectine-rijk materiaal van de middenlamella, opgespannen tijdens de vorming van intercellulaire ruimten, breekt tot wrat- en filamentvormige structuren. Een gedetailleerde studie van IPP's in het schorsparenchym van *Asplenium aethiopicum* (**hoofdstuk 9**) toonde aan dat, naast pectines, ook extensines aanwezig waren in deze structuren. Aangezien de celwandsamenstelling van IPPs

duidelijk afwijkt van die van zowel omliggende celwanden als midden lamellae, kunnen deze niet gevormd worden enkel door materiaal van de midden lamellae.

Collenchym, geëlongeerde cellen met verdikte primaire celwanden, werd voor zover gekend, nog niet voor leptosporangiate varens beschreven. Nochthans is een gelijkaardig weefsel aanwezig in de bladsteel van *Asplenium rutifolium* en verwante soorten. Om na te gaan of we dit weefsel als collenchym mogen aanduiden, werden de structuur, celwandsamenstelling en biomechanische eigenschappen van collenchymatische weefsels van *Asplenium rutifolium* onderzocht (**hoofdstuk 10**). Ondanks het feit dat onze resultaten een duidelijke gelaagdheid aantonen (zowel in structuur als in celwandsamenstelling, is het onmogelijk om met zekerheid te zeggen dat de collenchymverdikkingen secundaire celwanden zijn. Dit hoofdstuk eindigt dan ook met een bespreking van de problematische definitie van collenchym, gevolgd door voorstellen voor toekomstig onderzoek.

In **DEEL 4**, tenslotte, worden de algemene conclusies opgesomd, gevolgd door een aantal vooruitzichten voor (en voorlopige resultaten van) verder onderzoek (**hoofdstuk 11**).

