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

O. Leroux\textsuperscript{a}, F. Leroux\textsuperscript{b}, A. Bagniewska-Zadworna\textsuperscript{c}, J.P. Knox\textsuperscript{d}, M. Claeyse\textsuperscript{e}, S. Bals\textsuperscript{b}, R.L.L. Viane\textsuperscript{a,∗}

\textsuperscript{a} Pteridology, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, BE-9000 Ghent, Belgium
\textsuperscript{b} EMAT, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium
\textsuperscript{c} Department of General Botany, Institute of Experimental Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland
\textsuperscript{d} Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, UK
\textsuperscript{e} Nematology, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, BE-9000 Ghent, Belgium

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\section*{ABSTRACT}

Cell wall appositions (CWAs), formed by the 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.

\section*{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 wary to narrowly elongated structures formed at, and continuous with, the inner cell wall surface by the 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; Oriiani 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 and Shalla, 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 defense 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 and Lim, 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 (Colotille 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; Bouillard, 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 characterised 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 thin roots and long root hairs have limited or inconsistent mycorrhizal colonisation. Such facultative mycorrhizal associations are most common in leptosporangiate ferns, including Aspleniaceae (Brundrett, 2002). Moreover, epiphytic and epilithic, 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, epiphytic or epilithic, and occur from sea level to over 4000 m altitude. Most taxa are found in tropical montane rainforests and south-temperate regions, though some common in leptosporangiate ferns (Ogura, 1972). Cell

2. Materials and methods

2.1. Materials

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 C. Van den hende (CV)): A. abyssinicum (RV11186—Uganda), A. centraftricanum (EB392—Uganda), A. ceterach (CV250b—Cyprus), A. crinicaule (RV11304—China), A. currorii (RV sn—Ivy Coast), A. eliotilii (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. nudus (RV10620—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. vulkensii (RV7313—Kenya, RV11124—Uganda). One sample was fixed in the field: A. sertularioides (EB357—Uganda).

2.2. Histology

For light microscopy, roots were fixed in FAA (90% ethanol 50%, 5% acetic acid and 5% 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 Maité staining was performed following Leroux et al. (2011).

2.3. Immunohistochemistry

For transverse sections, roots of A. eliotilii were clamped in between two sheets 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 (Claussen et al., 2003; Verherbruggen et al., 2009); anti-galactan antibody LM5 (Jones et al., 1997), anti-arabian antibody LM6 (Willats et al., 1998), anti-xylan antibody LM11 (McCartney et al., 2005), anti-xylolucan antibody LM15 (Marcus et al., 2008), anti-(galacto)glucuronannan 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). Immunolabelling was performed as described elsewhere (Blake et al., 2006; Leroux et al., 2011; 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 512 x 512 pixels. Z-stacks were made every 0.5 µm and combined in one X/Y image.

2.4. Scanning electron microscopy

Longitudinal hand-cut sections of roots were fixed in FAA and processed following Leroux et al. (2011). Samples were photographed digitally using an EVO40 scanning electron microscope (Carl Zeiss, Germany).

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 4% formaldehyde and 2.5% 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-mm-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 200 kV.

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. 1a and 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., 2011, Fig. 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. elliottii* (Fig. 1a and 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. elliottii*, *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. 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. 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. 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 Fig. 1a and 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. 1d).

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. 2a) or elongated (Fig. 2b and c) and often branched (Fig. 2c) CWAs. We found CWAs both in between and on top of the helical cell wall thickenings (Fig. 2a). The surface of the CWAs was rather granular. Cells with CWAs (Fig. 2a, c, and d) frequently contained fungal-like structures. However, these are not necessarily responsible for the CWAs and some might be actinomycetes.

Transmission electron microscopy provided important additional information as it showed the presence of microbes in the cell walls as well as in most of the CWAs (Fig. 3a). In some cells fungal structures partially penetrated the cell walls (Fig. 3a). The
binding module recognizing crystalline cellulose (CBM3a). The LM21, and callose (Biosupplies 400-2) as well as a carbohydrate, xylan (LM11), xyloglucan (LM15), (galacto)(gluco)mannan (LM19), RG-I-related galactan (LM5), RG-I-related pectic arabinan (LM6), pectic homogalacturonan (JIM5, JIM7, JIM8), cell wall thickenings (Fig. 3d). The deposited material was often stratified (Fig. 3b), suggesting that additional cell wall material was laid down as the microbe was trying to escape the CWAs. Most CWAs were characterised by a distinct margin with a lower electron density (Fig. 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. 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. 3d). CWAs were not known in Aspleniaceae and similar structures have been described in few other ferns (Archer and Cole, 1986; Boullard, 1957). CWAs occurred in all Asplenium species investigated, either collected in the wild or cultivated outdoors or in the greenhouses of the botanical garden. 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. As we also found CWAs in roots fixed in the field, their occurrence is widespread and not limited to infections induced during cultivation.

CWAs were 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.

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).

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. 4a and 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. 4c and d). MAb with specificity for (1\(\rightarrow\)4)-\(\beta\)-d-galactan (LM5), (1\(\rightarrow\)5)-\(\alpha\)-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\(\rightarrow\)3)-\(\beta\)-d-glucan, was not detected in the CWAs. Both the phloroglucinol and Maüle tests failed to stain the CWAs.

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 diameter of the penetration pegs ranged from 0.1 to 0.4 \(\mu\)m. 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 immobilized 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. 3b). The deposited material was often stratified (Fig. 3b), suggesting that additional cell wall material was laid down as the microbe was trying to escape the CWAs. Most CWAs were characterised by a distinct margin with a lower electron density (Fig. 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. 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. 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. 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. 3e).

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CWAs were not known in Aspleniaceae and similar structures have been described in few other ferns (Archer and Cole, 1986; Boullard, 1957). CWAs occurred in all Asplenium species investigated, either collected in the wild or cultivated outdoors or in the greenhouses of the botanical garden. 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. As we also found CWAs in roots fixed in the field, their occurrence is widespread and not limited to infections induced during cultivation.

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![Fig. 2. Scanning electron micrographs of A. eelliottii roots. (a) Inner wall surface of cortex cell showing helical cell wall thickenings (arrow), numerous wart-like and some finger-like CWAs (arrowheads), and fungal structures (fs). (b) CWAs (arrowheads) predominantly occur on inner surface of outer tangential cell walls. (c) CWAs (arrowhead) co-occurring with fungal-like structures (fs). (d) Fungal-like structures (fs) in the epidermis. Scale bars: 6 \(\mu\)m.](image-url)
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.

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. In this study, we used an anti-β-1,3-glucan antibody and this epitope was not detected in CWAs of *A. elliottii*. Immunolabelling further indicated the presence of partially methyl-esterified pectic homogalacturonan, xyloglucan, mannan and crystalline cellulose in CWAs. These polymers also occurred in helical cell wall thickenings (Leroux et al., 2011), 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). However, negative phloroglucinol/HCl and Maüle tests confirmed by negative thioacidolysis reactions (Leroux et al., 2011) in outer cortex tissue containing CWAs, suggest the absence of lignins in CWAs of *A. elliottii*. In *Asplenium* roots all cell walls associated with CWAs were 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 in healthy plants, but can also be synthesised as a result of infection or wounding in order to increase resistance to fungal enzymes as well as constituting a stronger physical barrier preventing fungal penetration (Codignola et al., 1989; Yao et al., 2007). Rapid synthe-
sis of phenolic compounds and their subsequent polymerization in the cell wall have been suggested to be a first 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 papilla, 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. elliottii*. 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 depositions 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* (Hedden, 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 basidioymycete 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 et al., 1991), *Botrytis* (Archer and Cole, 1986), *Colletotrichum* (Mims and Vaillancourt, 2002; Wharton et al., 2001), *Erisyphe* (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), *Tri- choderma* (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 localised mechanical force or penetration, and/or a chemical stimulus caused by local dissolution of the cell wall at penetration sites.

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 micro-organisms which 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-pathogen interactions in ferns in general.

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