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Plant Cell Wall, a Challenge for Its Characterisation

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Abstract

The plant cell wall is a complex 3D network composed of polysaccharides, lignin and proteins. The knowledge of the structure and content of each cell wall polymer is a prerequisite to understand their functions during plant development and adaptation but also to optimise their industrial applications. The analysis of cell wall compounds is complicated by their multiple molecular interactions. In this review, we present numerous methods to purify, characterise and quantify proteins, polysaccharides and lignin from the wall. Two kinds of approaches are detailed: the first presents *in vitro* methods which involve the breakdown of the molecular linkages between polymers thanking to chemical, physical and/or enzymatic treatments. The second approach describes *in situ* methods that allow the cell wall polymer characterisation thanking to many analytical techniques coupled with microscopy. If microscopy is the common point of all of them, their development is associated with improvement of analytical techniques, increasing their power of resolution.

Keywords

Polysaccharides, Proteins, Lignin, Purification, Spectroscopy, Chromatography, Immunology, Microscopy, Plant Cell Wall

1. Introduction

Plants represent an important form of life on Earth. The organic carbon they produce by the fixation of atmospheric CO_2 during photosynthesis is the most abundant biological resource. Cellulosic biomass, a non-food carbon resource, can be found in agricultural and forestry products as well as in by-products (stalks, leaves, and

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husks, chips and sawdust from lumber mills), municipal waste (household garbage and paper products) and industrial waste (black liquor). Cellulosic matter is made up of acomplex three-dimensional network of cellulose/hemicelluloses embedded in a heterogeneous matrix of pectins and proteins. Secondary metabolites, lignin and mineral ions form the other components of the wall. Primary cell wall is found in all cells from algal to terrestrial plants. The tracheophytes clade exhibits, in some specialised cells such as fibres, vessels, tracheids and sclereids, an additional thicker secondary wall consisting of an interconnected network of cellulose and lignin [1]. As an exoskeleton, the extracellular primary or secondary walls give cells their shape. Moreover, the molecular compositions and the diversity of linkages within cell wall polymers define some new properties for plants such as mechanical and chemical resistance. As a consequence, the fine knowledge of its molecular composition is a prerequisite to understand how the polymers influence physicochemical and biological properties of the wall. However, the complexity of the plant cell walls makes their analysis difficult. Here we propose to review all of our knowledge on the analytical techniques, technologies or approaches developed for plant cell wall analysis.

2. Plant Cell Wall Composition

The most characteristic component found in all plant cell walls is cellulose accounting for 40% to 60% of the weight of the biomass. It consists of a collection of β-(1-4)-linked glucans (**Figure 1**) that interact through hydrogen bonds forming a semi-crystalline microfibril. In addition to cellulose, plant cell walls contain several other polysaccharides that can be grouped into hemicellulosic and pectic polymers. Depending on plants and tissues analysed, 20% to 40% of the carbon source are hemicelluloses. Hemicelluloses are a family of wall polysaccharides having β-(1-4)-linked backbones of glucose, mannose, or xylose termed as xyloglucans, xylans, mannans, galactomannans, glucomannans, galactoglucomanannans and β-(1-3,1-4)-glucans [2] [3]. Xyloglucans, which are found in primary cell walls of a wide number of higher plants, can be considered as the main hemicellulosic polymer (**Figure 1**). They have a cellulosic backbone to which α-D-xylose residues are linked at O-6

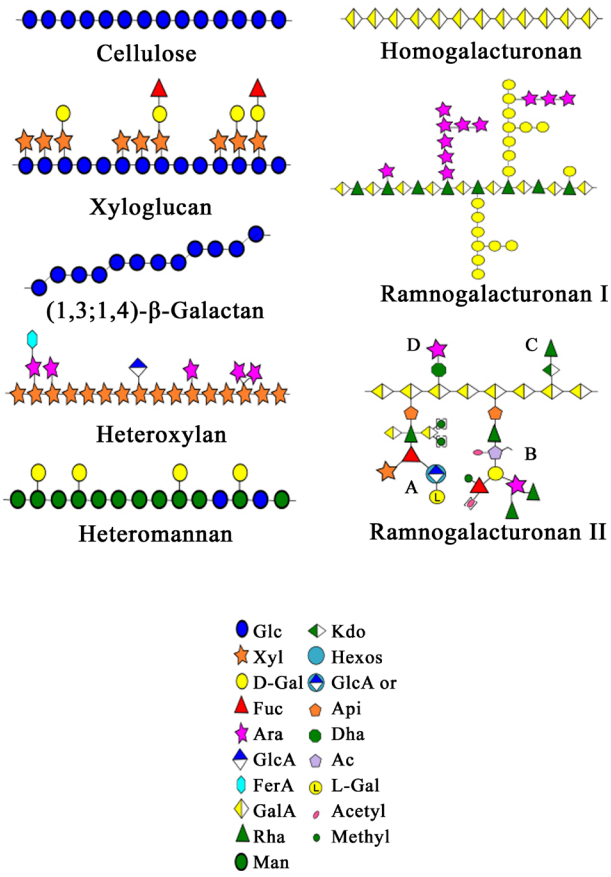

Figure 1. Schematic representation of some cell wall polysaccharides ([423] [424]).

and can be substituted at O-2 with *β*-galactose or *α*-L-arabinose. Other polymers complete the panel of the wall hemicellulosic polysaccharides. Xylans form a large group of polysaccharides with the common feature of a backbone of *β*-(1-4)-linked xylose residues (**Figure 1**). About 80% of the xylan backbone can be substituted with monomeric side-chains of arabinose or glucuronic acid linked to O-2 and/or O-3 of xylose residues, and also by oligomeric side chains containing arabinose, xylose, and sometimes galactose residues [3]. The arabinose substitution of xylan can be esterified by ferulic and diferulic acids [4]-[6] and can then be covalently linked to lignin monomers *via* an ether bond [7] [8]. Mannans and galactomannans (**Figure 1**) are mannose *β*-(1-4)-linked polysaccharides whereas glucomannans and galactoglucomannans are non-repeating glucose and mannose backbone polymers. Mixed *β*-glucans (MLG), essentially found in *Poales* [9] [10] are linear chains of repeat units of *β*-(1-4/3)-glucose linkages (**Figure 1**) giving plants specific motives for example G4G4G3G for *Equisetum genius* where G designs Glc and 3 or 4 the type of linkage [11]. Pectic polysaccharides are a covalently linked galacturonic acid family of polysaccharides including homogalacturonans, rhamnogalacturonans I (RGI) and rhamnogalacturonans II (RGII) [12] [13]. Homogalacturonans are unbranched homopolymer chains of a *α*-(1,4)-linked D-GalA making up more than 50% of the pectin (**Figure 1**). To form RGII, clusters of complex side chains are attached onto the O-2 or O-3 position in the galacturonan backbone (**Figure 1**). These side chains are composed of 12 different glycosyl residues (some of which are rare) linked together by at least 22 different glycosidic bonds. RGI is a branched polymer with a backbone of disaccharides (*α*-(1,4)-DGalA-*α*-(1,2)-L-Rha) repeats (**Figure 1**). The Rha residues in the backbone can be substituted with galactan, arabinan, and/or type II arabinogalactan side chains. Unlike RGII, the structure of the side chains of RGI can vary greatly amongst plants [14].

Lignin, which makes up 10% to 24% of the weight of biomass, is an aromatic phenylproprionate consisting of covalent linkages of three aromatic alcohol monomers differing in their degree of methoxylation: hydroxycinnamyl, p-coumaryl, coniferyl, and sinapyl alcohols. When monolignols are incorporated into lignin polymers, they become respectively p-hydroxyphenyl H, guaiacyl G, and syringyl S phenylpropanoid units. These units are linked together during a lignification process, thanks to radical coupling reactions. Structure and amount of lignin depends on taxa, cell types and cell wall layers and can be influenced by developmental and environmental phenomena [15]. Lignin contains energy and can be burned to produce steam and electricity for the biomass-to-ethanol process.

Cell wall proteins are ubiquitously found from algal to land plants andform strongly different groups of plant cell proteinsboth by their amino acid content (unusually rich in one or two amino acids, contain highly repetitive sequence domains) and their level of N- or O-glycosylation. The most abundant and studied plant cell wall proteins are the hydroxyproline-rich glycoproteins (HRGPs) or extensins, the arabinogalactan proteins (AGPs), the glycine-rich proteins (GRPs), the proline-rich proteins (PRPs), and chimeric proteins that contain extensin-like domains. Abundance of these proteins in the walls differs depending on the cell type. Thus, each can be assumed to have functions specific to its particular cell type. Extensins are said to be structural cell wall proteins that may also play a significant role in development, wound healing, and plant defence. As extensins, PRPs are thought to be insoluble in the cell wall matrix. PRPs are implicated in various aspects of development (germination to pod formation, early stages of nodulation) and the expression of PRP genes is influenced by wounding, endogenous and fungal elicitors, ethylene, drought, and light. Contrary to the former proteins, AGPs are not covalently linked to the cell wall and therefore do not have a structural function [16]. The majority of the characterised AGPs contains between 1% and 10% (wt/wt) proteins and more than 90% (wt/wt) carbohydrate. Many studies implicate arabinogalactan proteins in several biological processes of cell proliferation and survival, pattern formation and growth, and in plant microbe interaction [17]. GRPs are a class of structural wall protein which seems to play important roles in the development of vascular tissues, nodules, and flowers and during wound healing and freezing tolerance [16].

3. Plant Cell Wall Component Extraction

In order to analyse *ex-situ* cell wall components, their dissociation/destructuration/disorganisation from each other is a prerequisite for their analysis. Linkage breakdown between polymers can be done by chemical, enzymatic, physical or by a mixture of two or three of these proceedings. The challenge is then to release all the cell wall components without any change in their chemical composition. The plant cell wall disorganisation greatly depends on the number and type of linkages (covalent, hydrogen, ionic, electrostatic linkage) between all of

them. With some few exceptions, polymer isolation, or more precisely polymer enrichment in extracted fractions is usually done by chemical and/or enzymatic methods, which purify specifically one family of cell wall macromolecules. Here, we describe the different approaches used by a large number of research groups for partial purification of plant cell wall polymers, as some new methods using for example ionic liquid as cell wall solvent. For full access to proteome and glycome, sequential extraction proceedings have been developed. These approaches combine the methods described in the following pages and are used to collect enriched fractions of proteins weakly and strongly bound to the matrix, or enriched fractions of pectin and hemicelluloses as described by Vanzin *et al*. [18].

3.1. Protein Purification

Two groups of cell wall proteins (CWP) can be identified, the first is proteins formed only with amino acids and the second is glycoproteins, which have in addition, a non-amino acid component, usually a glycosidic fraction (2 to 15 carbohydrates) covalently linked to a hydroxyl and/or an amid function of some particular amino acid [19]-[22]. *Arabidopsis thaliana* genome analysis predicted that approximately 5000 genes encode proteins and/or glycoproteins targeted at the secretory pathway such as the wall compartment. It is probable that not all of them are CWP. Their amount, location and chemical environment require, for their isolation, the use of particular extractive buffers such as high ionic extractive salt buffers [23]-[25].

Depending on their linkage strength with the wall, CWP can be divided in labile proteins versus weakly bound proteins. Labile protein extraction can be done directly from liquid media of plants or cell cultures [26] [27], or by vacuum infiltration [28]. The extraction of weakly bound proteins (Van der Waals interactions, hydrogen bonds, hydrophobic or ionic interactions) can be done by salt solutions ($CaCl_2$, LiCl, NaCl) without detergent. After protein extraction, their precipitation is usually done with trichloroacetic acid (TCA), acetone, chloroform/methanol, ammonium sulphate or combinations of them [29]-[31]. If cell wall glycoproteins are preferred as the whole proteome isolation, they can also be isolated as one fraction of CWP or directly by lectin conjugated resin such as ConA [32]-[35], with specific resin, e.g. boronic acid-functionalised beads [36], by capture of hydrazide-based glycoprotein [37] and by β-Glc-Yariv precipitation [38] [39]. Enzymatic and carbohydrate modification allows highly efficient purification of glycoproteins [40]. As for proteins, glycoproteins capture can be performed by different strategies which can be adapted to plant material used to improve the coverage of the glycoproteome, as well as the quantification of glycopeptides [41]. CWP can then be stored at −80˚C before analysis.

3.2. Polysaccharide Purification

Three approaches can be carried out for plant cell wall polysaccharide purification. The first consists of a partial purification of one family of polysaccharides such as pectins, cellulose or hemicelluloses without paying any attention to the other cell wall components. These methods have been developed from a large panel of plants known as high producers of one of the cell wall polysaccharides. The second approach consists of a sequential extraction procedure suitable to collect numerous fractions enriched in one compound. The last uses physical or chemical processes suitable to solubilise cell wall components.

3.2.1. Cellulose Isolation

In literature, cellulose purification is usually obtained by elimination of other cell wall components. The dissolution efficiency of cellulose depends greatly on the ability of solvents to disrupt its interchain hydrogen bonds. Some powerful solvents can dissolve cellulose such as thiourea/H_2O, NaOH/urea, NaOH/CS_2, alkali/H_2O, LiOH/urea/H_2O, molten salt like $LiSCN{\cdot}2H_2O$ and $LiClO_4{\cdot}3H_2O$, N-methylmorpholine-N-oxide (NMMO), dimethyl sulfoxide (DMSO)/tetrabutyl ammonium fluoride (TBAF), LiCl/1,3-dimethyl-2-imidazolidinone (DMI), LiCl/N-methyl-2-pyrrolidine (NMP) and LiCl/N,N-dimethylacetamide (DMAc) [42] [43]. More recently, ionic liquids such as 1-butyl-3-methylimidazolium acetate ([Bmim]Ac) [43] have been used to dissolve cellulose [44]-[49]. If cellulose is the major component of the cell wall, its isolation/purification requires a preliminary treatment, for example with trifluoroacetic acid (TFA), to hydrolyse non-cellulosic polymers [50].

3.2.2. Pectins Isolation

Two groups of pectins can be found in the plant cell wall: low methylesterified pectins which are weakly bonded

to the matrix [51] and high-methylesterified pectins which can be termed labile pectins. Methylesterified pectins can be easily extracted by cold water [52] [53], or hot water [54]-[58]. Large amount of pectins can also be isolated by a hot acidified water known as conventional acidic extraction [59]. Pectins can also be extracted from an alcohol-insoluble solid phase as described by Bertin *et al*. [60]. Weakly bounded pectins, particularly those present in the middle lamella, can form gels due to Ca^{2+} bridges between two carboxyl groups belonging to two chains in close contact with each other [61]. Thus, Calcium removal by chelating agent can be considered another efficient method for pectin release [62]. Various chelating agents have been used such as ethylene diamine tetra-acetic acid (EDTA) [62], cyclohexanediamine tetra-acetic acid (CDTA) [63] [64], ammonium oxalate [54], sodium carbonate [55] [63] or sodium hexametaphosphate [52] [62] [65]. All methods presented so far, with the exception of CDTA method, undergo thermal degradation of pectin polymers [55]. As a pretreatment, microwave heating at ambient and moderate pressures, ultrasonication and a super-high frequency electromagnetic field may give increased extraction yield and pectin quality [66]-[77]. With these methods, the quality of pectins solubilised is improved due to the reduction of both time and temperature [78]. To have access to a more strongly binding pectin fraction, CDTA treatment can be coupled with sodium carbonate (0.05 M) treatment with or without sodium borohydride, known as a protectant for the carbohydrate's β-elimination [79]. But hemicellulosic polymers usually contaminate this last pectic fraction.

Enzymatic approach for depectination can be used either to purify non-modified pectins orto obtain degraded pectins (oligosaccharides, deesterified homogalacturonans...), depending on the enzymes activity. Pectinolytic enzymes are classified according to the nature of the substrate (pectin, pectic acid, oligogalacturonate), the degradation mechanism (trans-elimination or hydrolysis) and the type of cleavage (endo and exo) [80]. These enzymes can be divided into two main groups: pectinesterases (PE) or pectin methylesterases (PME) and depolymerases (polygalacturonases and lyases). PE or PME areexpressed in both plant, fungal and bacterial taxa [81]. They catalyse the hydrolysis of methyl esters of highly methylesterified pectin-bonds, causing the release of methanol and the formation of polygalacturonic acid [81]. The depolymerases are hydrolases (polygalacturonases and lyases) that have endo or exo-galacturonases activities. The depolymerases may be subdivided depending on the substrate and the cleavage mechanism of the glycosidic bonds in four categories: polygalacturonase (PG), polymethylgalacturonases (PMG), polygalacturonate lyases (PGL) and polymethylgalacturonate lyases (PMGL). The PG and PMG respectively act on the pectin by a hydrolysis mechanism, while PGL and GPLG act respectively by β-elimination of pectin [82]. Most commercial preparations of pectinases are of fungal origin, *Aspergillus niger* being the most commonly used source for industrial production of pectinolytic enzymes [83]. Thus, using the combined action of endoPG (*Aspergillus niger*) and PME (*Aspergillus aculeatus*), Pauly *et al*. [84] conducted a partial depectination of pea cell walls. Following the action of an endoPG (*Aspergillus aculeatus*, enzymes purified, without galactanase, galactosidase, arabinanase, arabinofuranosidase, xylosidase and esterase activity) on highly purified potato pulp, Byg *et al*. [85] obtained undegraded RGI with good yields of extraction. A mix or a sequential extraction proceeding can be used to recover all or one fraction of cell wall pectin.

3.2.3. Hemicelluloses Isolation

Because hemicelluloses are a family of polymers strongly linked to lignin and cellulose, their extraction is usually done by high concentration of alkali or organic solvents. Alkaline solutions hydrolyse the ester bonds and hydrogen linkages between polysaccharides and non-polysaccharides. That is why, extraction of hemicelluloses is usually done with aqueous alkali solutions such as potassium [86], sodium [87], lithium [88] or calcium hydroxide [89], and hydrogen peroxide [90]. But to minimise the reducing end degradation of hemicellulose, sodium borohydride can be added [91]. KOH has been demonstrated to be the most efficient base to isolate heteroxylans from corn bran [92] whereas NaOH is a better solvent for glucomannans [91]. (Galacto) glucomannan isolation can be facilitated by the addition of borates which form anionic products, having a higher solubility in alkali solutions. By using a gradient of concentration of alkali solution, different hemicellulosic polymers can be isolated [91]. Without prior delignification, hemicelluloses from *H. ammodendron* and *E. angustifolia* have been extracted with ethanol/H20 under acidic conditions and hydrogen peroxide under alkaline medium [93]. This treatment allows solubilisation of hemicelluloses and lignin, and authors found no significant degradation or oxidation of hemicelluloses after alkaline peroxide post-treatment. Therefore, they deem it possible that lignin has a protective effect on them. However, not all the hemicellulosic substances were solubilised. According to mentioned authors, the hemicelluloses which remain are probably tightly bound to the cellulose [93]. Delignifi-

cation and depectination increase the yield of hemicelluloses extracted [55]. Few neutral solvents have been used, among them dimethylsulfoxide (DMSO) [94]. Hemicellulose-DMSO extraction has the advantage of keeping the acetylation of xylans that is saponified by extraction with alkali. This extraction method thus provides low-modified hemicellulose fractions, but has the disadvantage of having relatively low extraction yields (<50%) [91]. Other solvents can also be used to extract hemicelluloses for example water or ethanol. By using pressurised low-polarity water and pressurised aqueous ethanol, Buranov and Maza [95] managed to remove 90% and 80% of hemicelluloses, respectively, from flax shives.

As for pectin, the yield of hemicelluloses extracted with chemical methods can be improved by physical pretreatments such as ultrasound [96], microwave irradiation [97] or steam pretreatment [98]. Partial hydrolysis after enzymatic treatment represents another way for direct analysis of soluble hemicellulosic oligosaccharides by mass spectrometry [84]. Two kinds of enzymes can breakdown hemicelluloses: the exohydrolases that release terminal monosaccharide units from the reducing end and the endohydrolases that cleave glycosidic bonds at random or specific positions [99]. Moreover, for each hemicellulosic polymer, enzymes involved in the cleavage of main chains and lateral chains of polysaccharides are necessary [100]-[103]. Hemicellulosic oligosaccharides can also be extracted from the biomass by physical process alone such as stem explosion or microwave treatment [104].

3.3. Lignin Purification

Two main kinds of method are used to isolate lignin: acidolyse methods [105]-[108] and enzymatic methods [106] [109]-[111]. Even if acidolysis methods are quite fast and allow high pure lignin to be extracted, they lead to structural modifications of these polymers [106] [112]. To limit these alterations, it is possible to decrease the acidity of dioxane solutions for example, although this usually results in a higher carbohydrate contamination [106] [109]. The enzymatic method consists of a biomass treatment with cellulolytic enzymes to hydrolyse polysaccharides and liberate lignin. This method leads to good purification yields and lignin undergoesonly minimal structural modifications [112]. However, isolated ligninis contaminated by carbohydrates (12%) [111]. A combination of these two methods, a cellulolytic enzyme treatment followed by a mild acidolysis, triggers high lignin yields with fewer impurities than the enzymatic process alone [106] [109] [110] [113].

Using a sequential extraction protocol, Sun *et al*. [114] purify lignin and hemicelluloses at the same time with mild dioxane, acidic dioxane, DMSO and alkali treatments. They obtained good yields of the two polymers and were able to find important structural features of lignin and hemicelluloses from barley straw. Ionic liquids, such as [Bmim]Cl (1-Butyl-3-methylimidazolium chloride) [115], [Amim]Cl (1-Alyl-3-methylimidazolium chloride) [116], [Bmim]MeSO4 (1-Butyl-3-methylimidazolium methylsulfate) [117], allow the dissolution of biomass and the regeneration of lignin, hemicelluloses and cellulose fractions without prior purification treatments (see “Cell wall destructuration” paragraph).

3.4. Cell Wall Destructuration

Another strategy for plant cell wall composition analysis is the destructuration of the biomass. Nevertheless, wood dissolution is virtually impossible because of high levels of organisation of the cell wall polymers [118]. Chemical and physical proceedings have been developed to breakdown polymer linkages producing full cell wall disorganisation. Free polymers can then be solubilised before their use or analysis.

3.4.1. Chemical Dissociation

The majority of known solvents have been developed for the solubilisation of cellulose, but present little effect on lignocellulosic biomass [119]-[124]. Biofuel development has highlighted ionic liquids as revolutionary solvents for biomass disorganisation [44]. Ionic liquids (ILs), considered as “green solvents”, are salt solutions with a melting point below 100˚C [125] [126]. The interest of ILs is based on their outstanding abilities to dissolve monomeric or polymeric compounds, polar or non-polar organic or inorganic compounds [127]. It has thus been shown that they can dissolve some polysaccharides such as chitin [128] or cellulose [44]. At the same time, Vesa and Reijo [129] were the first to demonstrate that it was possible to dissolve more complex matrices such as wood completely in an IL (1-butyl-3-methyl imidazolium chloride [BmimCl]). The following work has sought to improve the biomass dissolution [130] and to understand the impact of cation and anion on the ability of IL to dissolve large amounts and diversity of lignocellulosic materials [46] [115] [131]-[133] (**Figure 2**).

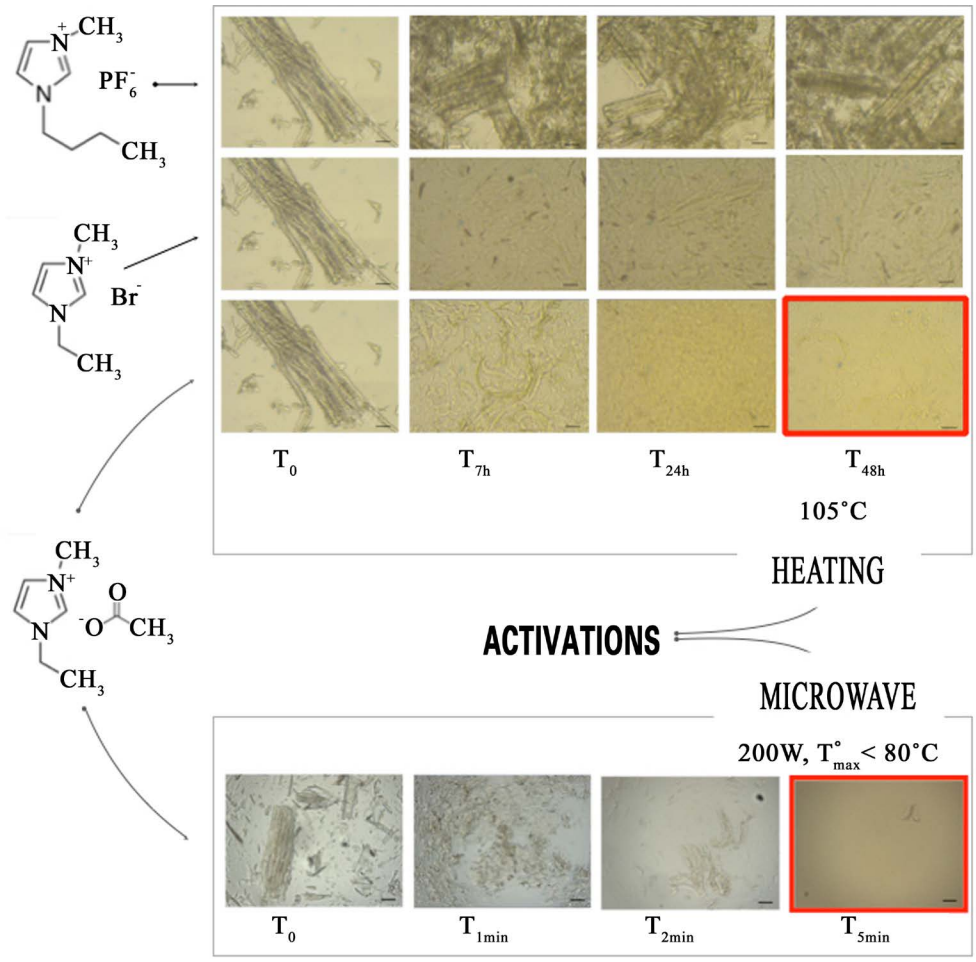

Figure 2. Douglas fir wood dissolution in presence of 3 different ionic liquids under constant heating and microwave activation (Plazanet and Costa, not published).

Wood destructuration by [EmimOAc] can be monitored by laser confocal fluorescence as demonstrated for switch grass [134]. Because of the numbers of polymeric linkages between cell wall polysaccharides, different combinations of ILs can be developed. Muhammad *et al*. [118] suggests that ILs having high hydrogen bond basicities ß are suitable solvents for wood biomass dissolution. One limitation of ILs efficiency is their hygroscopicity requiring the use of dry material [135]. A new solvent, tetra-n-butylphosphonium hydroxide ($[P_{4,4,4,4}]OH$) was suitable to dissolve cedar wood in aqueous solution at 60˚C while being stirred gently for 24 hours. However, in this method the solution induced a partial lignin degradation [135].

3.4.2. Physical Dissociation

Physical processes including comminution (mechanical reduction in biomass particulate size), steam explosion (heating by high-pressure) and hydrothermolysis (solvolysis by hot compressed liquid water) have been applied for biomass disruption. But all these techniques strongly destroy the biomass, and they cannot be used for cell wall analysis. These methods, considered as pretreatment are able to break the lignin seal and disrupt the crystalline structure of cellulose [136]. Biomass can also be disrupted by ultrasonic (UAE) and microwave-assisted extraction (MAE) [137] [138]. These two techniques are suitable to extract large amounts of polysaccharides in shorter time, with less solvent than Soxhlet extraction [139]. But parameter control and temperature elevation during the extraction procedure are the two main limits for a large application of UAE and MAE. Some combinational methods have then been tested such as ultrasonic-microwave synergistic extraction (UMSE) [140] and enzymolysis-ultrasonic assisted extraction [141]. They have been developed to increase purity and yield ofextracted polysaccharides [142].

4. *In Vitro* Plant Cell Wall Component Analysis

Analytical proceedings are largely dependent on the chemical composition and the amount of the bio-molecules to be characterised.

4.1. UV/Visible Spectrometry Methods

4.1.1. Protein Quantification

Several colorimetric methods exist to determine protein concentration in solution: the Biuret [143], the Lowry method [144], the bicinchoninic acid (BCA) assay [145], the Coomassie Blue G-250 dye-binding assay [146], the colloidal gold protein assay [147], two dye-binding protein assay using Pyrogallol Red [148] or Pyrocatechol [149], and the Pierce 660 nm assay [150].

4.1.2. Polysaccharide Quantification

Polysaccharides are converted to furfural or a derivative of furfural by strong acidic solutions. Subsequent, complexion of the furfural or its derivative with an appropriate organic developer provides the formation of a chromophore, the absorbance of which, in visible light, allows the quantification of monosaccharides. Organic developers used to quantify total, neutral monosaccharides or uronic acids are mainly indole [151], orcinol [152], carbazole [153] [154], resorcinol [155], m-hydroxydiphenyl (m-HDP) [156] or phenol [157]. The phenol-sulphuric acid method is said to be the easiest and most reliable method to quantify neutral sugar because of its sensitivity and simplicity [158]. The m-hydroxydiphenyl (m-HDP) method is the most efficient to quantify uronic acids [156]. Due to the interference of uronic acids in the determination of neutral monosaccharides and vice versa, a correction patch should be applied as described by Montreuil and Spik [159]. Some of the methods described above have been miniaturised to gain greater sensitivity and to reduce reagents, biomass and time. Among them, we quote a micro-scale phenol-sulphuric assay [158] and a micro-scale m-HDP assay [160].

4.1.3. Lignin Quantification

It is also possible to quantify lignin and some secondary metabolite content thanks to the use of UV spectrophotometry techniques. The most common methods to quantify lignin are the acetyl bromide, thioglycolic acid, and Klason. The first two methods are based on solubilisation of lignin and determination of absorbance values at 280 nm whereas the Klason mathod is a gravimetric assay. In the acetyl bromide method, lignin is first solubilised in acetyl bromide before their quantification at 280 nm [161]-[164]. The thioglycolic acid method is based on the formation of thioethers of benzyl alcohol groups found in the lignin, resulting in solubilisation of this polymer under alkaline conditions. The acetyl bromide method is faster, simpler and presents better recovery of lignin than the other methods [165]. Another simple method to quantify lignin from lignocellulosic biomass has been developed by Kline *et al*. [166]. After the dissolution of the whole biomass in an ionic liquid ([bmim]Cl), absorbance of the solution is read at 440 nm via UV-VIS spectrophotometry. Lignin concentration is then calculated using an extinction coefficient from standard lignin (isolated from biomass bythe Organoslov process) with Beer-Lambert Law. Coupled with multivariate analysis, fluorescence spectroscopy was used to develop models to study different parameters of poplars and Northern red oak such as total lignin, extractive and estimation of total holocellulose content [167] [168].

4.2. Raman and Infrared Spectroscopies

Raman and infrared (IR) spectroscopes are vibrational spectroscopy techniques used to identify complex plant molecules [169]. The Raman spectrometry induces a change in the polarisability of the molecule whereas the IR transition exhibited a change in the dipole moment of polar components. These two non-destructive techniques are complementary: Raman spectroscopy is best at vibration of non-polar groups whereas IR spectroscopy is best at asymmetric vibration of polar groups. Vibrations which are seen in both spectra serve as evidence in the case of complex samples [170] [171]. Each polymer can be characterised by vibrational transitions of known chemical groups and a vibrational fingerprint of the molecule [170] [171]. A chemical function of a polymer is characterised by one or several wavenumber(s). For cellulose, these vibrational spectrometries allowed the identification of the different types of cellulose [172]-[175], the determination of its crystallinity [175]-[178] and the chemical groups orientation inside the main chain [179]-[181]. Hemicelluloses [182]-[186] and pectic polysac-

charides [187]-[193] have also been structurally characterised from several plant species. Synytsya *et al*. [193] showed FT-IR spectroscopy was more adapted to study functional groups of pectin (hydroxyls, carboxyls...), whereas FT-Raman spectroscopy was better to analyse the structure of the main chain (*i.e.* glycosidic bounds). Thus, FT-IR spectroscopy is an interesting tool to determine the degree of methylesterification of pectin [190] [191]. Kacurakova *et al*. [194] studied model plant cell wall polysaccharides by FT-IR spectroscopy in order to establish model data allowing identification of pectic polysaccharides and hemicelluloses [194] [195]. Raman and IR spectroscopes are also suitable to characterise lignin [196]. Total lignin content can be estimated by FTIR [197], NIR [198], UV-Raman [199] and FT-Raman [200]. FT-Raman [201] [202], UV-Raman [203] [204] and FTIR [205] have been used to determine S/G/H content and to analyse the lignin structure [203] [204] [206] [207].

4.3. Mass Spectrometry

The mass spectrometry (MS) has often been used instead of conventional detectors coupled to plant cell wall polymer separation. But MS can also be used directly on purified polymers.

4.3.1. Protein Fingerprint

Mass analysis is usually done after electrophoretic or liquid/nano-liquid chromatographic peptide separation. Matrix Assisted Laser Desorption/Ionisation Time Of Flight (MALDI-TOF-MS) and Electrospray Ionisation Time Of Flight (ESI-TOF-MS), that differ by their method of ionising analysts, are the two most conventional mass spectrometry technologies referenced in literature [208]-[212]. MALDI-TOF-MS is mainly done for peptide identification purified in 2D-gel electrophoresis before trypic in-gel digestion [213]. Electrospray, usually coupled with nano-Liquid Chromatography (LC), is suitable for the analysis of large numbers of proteins especially for membrane associated proteins [214] [215]. In some cases, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and LC can be combined for proteome analysis on an approach named GeLC-MS [215]. But until now, this approach has not been carried out for CWP. *De novo* protein sequencing from unknown or poorly known genomes and analysis of peptides containing non-proteinic or modified amino acids have also been developed using peptide fragmentation. Edman techniques (cleavage of the amino-terminal labelled residue) for protein sequence determination have been replaced by MS/MS proceeding: LC-ESI-MS/MS and more recently MALDI-TOF-MS/MS [216]. Peptide purity for fragmentation is a prerequisite for high quality sequence and explains why LC-MS/MS is preferred to gel-MS/MS. Electron capture dissociation (ECD) and Electron Transfer Dissociation (ETD) are promising new fragmentation methods [217] applied for the identification of AGP31, an *A. thaliana* cell wall O-glycoprotein. Mass spectrometry can be used for proteomic quantification. Two methods have been developed: label-free and stable isotopes labelling methods [215]. Isobaric tags for relative and absolute quantification (iTRAQ) and isotope-coded protein labelling (ICPL) [218] are applied to plant proteomic [219] [220] after incorporation of stable modified amino acid to the culture media (SILAC) or under ^{15}N isotope flux [221]-[223].

4.3.2. Polysaccharide Fingerprint

Different types of MS have been used for structural cell wall carbohydrates determination such as MALDI-TOF, ESI [224]-[226], Ion Trap Mass Spectrometry (ITMS) coupled with either MALDI or ESI [227] and Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) [228]. In Oligosaccharide Mass Profiling (OLIMP), oligosaccharides are released by specific glycosylhydrolases. This method combines the advantages of enzymes specificity and the high sensitivity of mass spectroscopy [229]-[231]. Coupled with micro-dissection, the technique is suitable to analyse pectin and xyloglucan structures in one cell type, such as the outer or entire epidermis cell layer, palisade mesophyll cells, and vascular bundles [231].

4.3.3. Lignin Fingerprint

MS can be used to analyse lignin structure from lignin degradation products or from whole lignin macromolecules. It is possible to degrade lignin by thermal or chemical treatments. For the first, rapid pyrolysis (py) techniques are the most commonly used to produce molecular fragments that can be analysed by MS or GC (Gas chromatography)-MS [232]. Py-GC-MS was widely used to characterise S/G/H ratio of lignin [233]-[235]. This technique needs small amounts of sample and the sample does not require pre-treatment except grinding. For the lignin study, several ionisation modes have been used with in-source py-MS [232]. The chemical methods to

degrade lignin include alkaline nitrobenzene oxidation [236]-[238] and cupric oxide [239] which combined with GC-MS give information on the minimal quantities of uncondensed phenylpropanoide units and their relative amount; and the permanganate oxidation [240], the hydrogenolyse [241], the thioacidolysis [242], the acidolysis [243], the ozonolyse [244] and the Derivatisation Followed by Reductive Cleavage (DFRC) method [245]. The analysis by GC-MS of the degradation products obtained using the latter methods provides structural characterisation of lignin. The thermochemolysis with Tetramethylammonium Hydroxide (TMAH) combined with GC-MS is a rapid method for the characterisation of the S/G/H ratio in plant material [246]. Structural characterisation and molecular weight distribution of lignin can be provided from non-degraded macromolecules by means of soft-ionisation mass spectrometric techniques (mainly MALDI-MS and ESI-MS) [247]-[251].

4.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

Even if NMR methods are less rapid and not as sensitive as others spectroscopic methods, they can provide useful structural information on the cell wall polymers [252].

4.4.1. Polysaccharide Fingerprint

NMR methods are too insensitive to use at the cellular level and must be applied from a sample resulting from a homogenising of several cell types [252]. NMR spectroscopy has been used to provide information on the chemical structure of plant cell wall polysaccharides and their interaction [252]-[260]. CP-MAS (Cross Polarisation Magic Angle Spinning) ^{13}C-NMR has been largely used to characterise cellulose crystallinity [261]-[264]. Structural polysaccharide information by NMR can also be available from the dissolution of the whole cell wall without the need to isolate fractions [252] [265]-[273].

4.4.2. Lignin Fingerprint

The molecular structure of lignin has been widely studied by NMR [274]. NMR techniques can be suitable as a qualitative and quantitative method forcharacterisation of lignin isolated or in the entire dissolved plant cell wall [275]. NMR technique has been used to quantify the S/G/S ratio in different biomass samples [276] [277], estimate the total lignin content [278] and analyse the lignin structure [279].

4.5. Electrophoretic Methods

4.5.1. Protein Fingerprints

The size of the putative cell wall proteome and the diversity of CWPs require the use of a combination of separative and analytical methods without any certainty as to the recovery of the whole wall proteome. The CWP analysis is classically done by 1D and 2D-PAGE electrophoresis followed by mass spectroscopy identification. The mass of tryptic digested peptides, given by MALDI-TOF MS or MALDI-TOF/TOF [280] are then mapped against databases prior to protein identification. The high amounts of acidic and basic CWPs like the occurrence of heavily glycosylated CWPs require some particular electrophoretic or chromatographic methods. The separation of basic proteins on 2D-PAGE can be achieved by blocking the cysteine oxidation during protein separation. Cysteine alkylation with iodoacetamide, acrylamide [281] [282], maleimide [283] or by disulphide exchange with dithiodiethanol [284] greatly increases the protein resolution in 2D-PAGE.

4.5.2. Polysaccharide Fingerprints

Carbohydrate gel electrophoresis method (PACE) with derivatisated reducing end sugar by fluorophore, such as 8-amino-naphtalene-1,3,6-trisulfonic acid (ANTS), has been developed for N-glycan [285]-[287] and cell wall polysaccharide analysis [288] [289]. The fluorophore-assisted carbohydrate electrophoresis (FACE) uses a combination of chemical and specific hydrolases which are able to release monosaccharides before their electrophoretic separation [290] [291]. Derivatisated glycans can also be analysed by capillary electrophoresis (CE) [292]. Unknown glycan migration can then be compared with standard if they are available. Mass spectroscopy (MS) coupled with CE (CE/MS) is no longer dependent on the standards used to identify simultaneously, the oligosaccharide and its molecular design [293]-[296].

4.5.3. Lignin Fingerprint

A method coupling capillary electrophoresis (CE) with on-column UV-detection has been developed to quanti-

tatively analyse phenolic lignin degradation products [297]. Isotachophoretic separation is another electrophoretic method used to separate biomass hydrolysis products such as phenol compounds [298].

4.6. Chromatographic Methods

Chromatographic methods, such as liquid and gaz chromatography, are the most conventional analytical processes developed for glycoprotein and glycan analysis. These separative methods can be coupled with a large panel of detectors: UV/visible, fluorescence, Pulsed Amperometric Detection (PAD) and/or mass spectroscopy.

4.6.1. Protein Fingerprints

Because glycoproteins are poorly separated by 2D-PAGE, LC coupled with tandem MS/MS are preferred. Tryptic digestion is coupled with a peptide N-glycosidase from sweet almonds (PNGase A) for N-glycans release before MS analysis [299]. For O-glycoproteins such as cell wall AGP previously purified by β-Glc-Yariv, their separation can be performed by 2D High Pressure Size Exclusion Chromatography (HPSEC) coupled with a double fluorescence and 215 nm UV detection [300]. Collected AGP fractions can be then deglycosylated with hydrogen fluoride or trifluoromethanesulfonic acid [301] followed by tryptic digestion before MS protein analysis.

4.6.2. Polysaccharide Fingerprints

Previously solubilised polysaccharides can be analysed from partial purification and enrichment fractions or directly after biomass disorganisation by chemical and or physical techniques. After their hydrolysis in monosaccharides, High Performance Liquid Chromatography (HPLC), GC or GC-MS can be used to determine the polysaccharide composition. LC-MS is also suitable to analyse oligosaccharides produced after a partial digestion of polysaccharides. The most common approach consists of the analysis of the monosaccharides composition of cell wall polysaccharides after they have hydrolysed or methanolysed [302]-[304]. For proteoglycans, a preliminary chemical deglycosylation-step can be done before analysis (see paragraph on Protein fingerprints). The monosaccharides are released from poly- or oligosaccharides with strong acid solutions such as trifluoricacetic acid usually coupled with high temperature. Such processes have been demonstrated to be efficient for cellulose depolymerisation, but may also lead to degradation of some hemicellulosic polysaccharides, such as xylose [305]. The methanolysis procedure is preferred because of its low level of monosaccharidedegradation [306]. Methanolysis releases stable methyglycosides and methylglycuronosides which can be derivatisated by pertrimethylsililylation [307] acetylation [308], trifluoroacetylation [309], reduction/acetylation [310] oxilmation/acetylation [311] and O-methyloximation/acetylation [312]. Volatile compounds can then be separated by GC or GC-MS. Underivatisated monosaccharides can also be separated by HPLC, but the sensitivity and the efficiency of the separation is lower than with the GC technique.

In some case, oligosaccharides and deglycosylated N-glycans can be directly identified by High-Performance Anion-Exchange Pulsed-Amperometric Detection (HPAE-PAD) and LC-MS [313]-[315]. Thin layer chromatography (TLC) is also a technique used to rapidly separate and estimate the content of mono- or oligosaccharide mixtures [316].

4.6.3. Lignin Fingerprint

GC has been used to determine the S/G/H ratio of lignin after their oxidation by cupric oxide [296] [297] [317] [318], permanganate [297] or alkaline nitrobenzene oxidation [237] [238] [319]. Coupled with MS, GC was applied to study lignin monomers obtained after thioacidolysis [242] or acidolysis [243]. It is also possible to analyse acidolysis products without derivatisation with HPLC, but with a lower resolution [243]. High-performance size exclusion liquid chromatography coupled with different detectors (multi-angle laser light scattering (MALLS), quasi-elastic light scattering (QELS), interferometry refractometry (RI) and UV detection) can be used to determine the molecular weight distribution of purified lignin [320].

4.7. Immunological and Immunological-Like Methods

Immunological techniques are used for polysaccharides, glycoproteins and protein identification and quantification. Generally monoclonal antibodies (mAbs) have been developed from neo-glycoprotein immunisation procedures or from retrospective characterisation after immunisation with cell-wall material [321] [322]. A lot of

mAbs recognise epitopes of RGI [323]-[329], homogalacturonan [330] [331], xyloglucan [332]-[334], xylogalacturonan [335], xylan [336], even epitopes of wall proteins such as AGP [337]-[340] and extensins [341]. This collection of mAbs has been completed by Pattathil *et al*. [342] with the creation of 130 new glycan-directed mAbs. Cell-wall-directed mAbs available are inventoried in CarboSource (http://www.ccrc.uga.edu/~carbosource/CSS_home.html), PlantProbes (http://www.plantprobes.net), Biosupplies Australia (http://www.biosupplies.com.au) and the Wall-BioNet (http://glycomics.ccrc.uga.edu/wall2/antibodies/antibodyHome.html). Once the mAbs recognising the epitope of the polymer of interest is bound, the surplus of mAb is washed, and a secondary antibody hybridised on the first one. The secondary antibody can be tagged with an enzyme, a radioisotope, a fluorochrome or a paramagnetic bead [343].

Carbohydrate-Binding Modules (CBMs) are discrete protein modules present in microbial or plant carbohydrolases (and few from non-hydrolytic proteins) and have a wide range of binding specificities toward cell wall carbohydrates [344]-[346]. Coupled directly with fluorophore (FITC) or with His-tag, CBMs can recognise cellulose [335]-[339] [347], xylans [348]-[350], and mannans [351] [352]. McCartney *et al*. [353] used CBMs containing His-tags in a three-stage procedure, with an anti-His antibody produced in mice (secondary stage) recognised by an anti-mouse FITC (tertiary stage). Because they deemed the available anti-His FITC antibodies less effective, they decided against a two-step procedure. Compared to antibodies, CBMs have several advantages. Firstly, because of their high specificity and small size compared to antibodies, CBMs can access their targetmore easily [351]. Secondly, gene or protein sequences of CBMs can be obtained, and the engineering of CBM specificities is possible once the protein structure is known [354]. Extracted carbohydrates can be spotted on several supports like membranes (Dot-blots; [355]), silica plates [356] or in different ELISA (Enzyme-Linked Immunosorbent Assay) plates [342] [357] before their analysis. However, a limit of these techniques is the efficiency of hybridisation between mAb or CBM with their target due to masked epitopes by other cell wall polymers or substituents, or by a polymer aggregation [311] [330] [333]. Moller *et al*. [358] have developed a technique named CoMPP for Comprehensive Microarray Polymer Profiling, in which glycans are sequentially extracted from plant tissues to generate microarrays. Microarrays are then probed with mAbs or CBMs specific to cell wall polymers. This technique allows them to compare *Arabidopsis* mutants providing a global snapshot of their cell wall composition. Immunological and immunolical-like methods can also be carried out on plant biomass dissolved in ionic liquid to rapidly obtain its main composition [359].

5. *In Situ* Plant Cell Wall Components Analysis

As prospective approaches, plant cell wall components can be directly identified and quantified inside the cell wall by specialised microscopic techniques. Specific dye reagents, protein-polymer recognition (antibodies, lectin, etc...), fluorescent and vibrational excitation and more recently mass spectrometry are susceptible to be used at a resolution level of µm or lower. For all these approaches, sample preparation, microscopic resolution and cartography reconstitution are the principal limitations for their large scale use. Probably one of the strongest difficulties is the sample preparation (quality and fitness of the section, flatness of the sample, component accessibility formeasuring).

5.1. Staining Microscopy

Tissue sections from 10 to 50 µm canusually be stained, in 10 min, by toluidine blue O [360] (**Figure 3(c)**) as a basic for cell labelling. To have an idea about the relative proportion of cellulose and lignin, wood sections can be sustained by the simultaneous action of safranin and alcian blue according to Tolivia and Tolivia [361] (**Figure 3(b)**). For finer knowledge of lignin composition and distribution in secondary cell walls, some specific dyes such as phloroglucinol-acid [362] (**Figure 3(c)**) and Maüler eagent [363] can be applied (**Figure 3(d)**).

5.2. Fluorescent Microscopy

Fluorescent and confocal fluorescent microscopes are usually used for the determination of the spatial distribution of target compounds by a large panel of chromophores. Safranine, known as a suitable lignin stain, produces a green/yellow fluorescence in the secondary cell wall and a red/orange fluorescence in the middle lamella (ML) region [364]. Other fluorescent stains generally used to localise lignin (even if there are non-specific) are

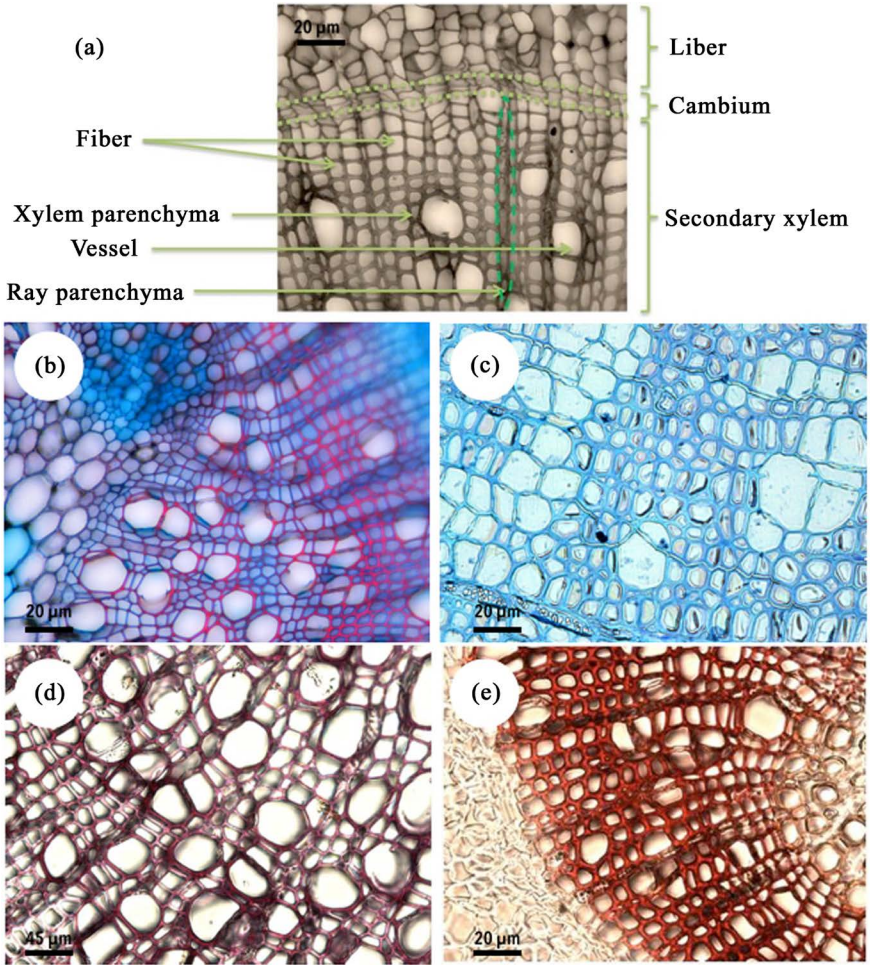

Figure 3. Transversal section of *in vitro* poplar wood. (a) Poplar wood organisation; (b) FASGA staining; (c) Toluidine staining; (d) Phloroglucinol staining; (e) Maüle staining (Costa, not published).

acriflavin [365], basic fuchsin [366] and berberine sulphate [367]. Contrary to lignin, cellulose is not an intrinsically fluorescent molecule. However, even after the removal of lignin, fluorescence can still be observed in different cellulose samples [368], probably because of proteins and residual lignin [369]. Fluorescent markers, such as 7GFE, calcofluor, S4B have been tested [370] [371] for their potential to allow imaging of cellulose distribution in the wall of *Arabidopsis* seedlings. Hoch *et al*. [372] have used pontamine Fast Scarlet 4B (S4B) to determine the cellulose distribution.

Wall polysaccharides can also be studied thanks to glycan-directed probes such as antibodies, carbohydrate-binding molecules (CBM) and lectins coupled with fluorophore (GFP, CFP, YFP, FICT) (**Figure 4(a)**). These techniques allow us to study the cell wall microstructure and polymer localisation *in situ* within complex plant tissues [373]-[377]. Multiple fluorophore dyes can also be used for simultaneous multiple compound identification. This approach is limited by the number of chromophores available and by the autofluorescence of some natural plant cell wall components. For fluorescence, mAbs, lectins and or CBMs are the same as those described earlier (compare the paragraph on Immunological and immunological-like methods).

5.3. Multi-Photonic Microscopy

Multiphoton laser scanning microscopy is another approach for three-dimensional fluorescence imaging. Two-photon and multi-photon excitation reduce light scattering, autofluorescence, photo bleaching and photo damage of living cells better than wide-field fluorescence or confocal microscopy [378]-[382]. Two-photon excitation in the NIR spectral region (740 - 1200 nm) has been applied for root tip analysis [383], embryos [384],

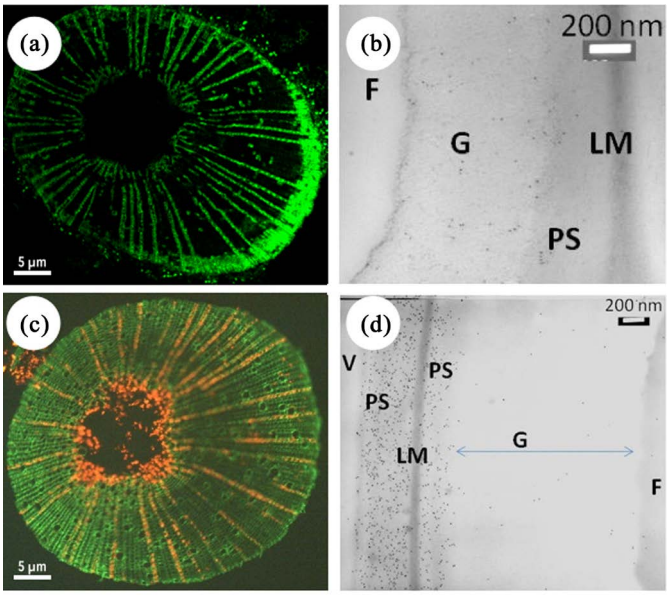

Figure 4. Transversal section of *in vitro* poplar wood. ((a), (b)) JIM8 immunolabelling observed by fluorescence (a) and by TEM (b); ((c), (d)) LM11 immunolabelling observed by fluorescence (c) and by TEM (d) (Costa, not published).

chloroplasts [385] and for intra-tissue nanodissection of cell walls [386]. A modified periodic acid schiff treatment using propidium iodide as cell wall stain gives a fluorophore suitable for monitoring plant cell wall evolution [387]. Monolignols, suchas ferulic acids that are a key molecular bridge between hemicellulose and lignin, are also two-photon excitable fluorophores [388]. However, until now, no application of multi-photon laser scanning microscopy has been published in literature.

5.4. Electronic Microscopy

Two electronic microscopes have usually been applied for plant cell wall component identification: Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). If SEM gives information about the cell wall surface, it can be coupled withelementary analysis for the identification of some particular incrustation inside the wall. For high-resolution SEM the sample must be made conductive, which is normally accomplished by coating biological specimens with vaporised metal or carbon. It is believed that these sample preparation procedures used for the plant materials can damage and ultimately change the native structure of the plant cell wall. With the recent introduction of high-pressure freezing and freeze substitution methods for preserving cells for infrastructural analysis, electron microscopy has become a valuable tool for correlative imaging [389]. However, there are still limitations with these imaging techniques and often a reduction of resolution is the resulting compromise. For *in situ* plant cell wall component identification, immunostaining of ultrathin sections of wood were incubated with a similar set of antibodies as those described previously (see paragraph on immunological and immunological-like methods). Sample preparation involves tissue embedding into a resin (LR-white for example). The secondary antibody is tagged with electron-opaque particles (**Figure 4(b)**).

5.5. Infrared and Raman Micro-Spectroscopies

IR and Raman micro-spectroscopy are suitable for analysis at the single cell wall level [390] [391] (**Figure 5**). FT-IR microspectroscopic imaging or FT-IR microscopy is able to locate plant cell wall polymers [392]-[394]. In some applications such as biorefinery, FT-IR microscopy is able to monitor the enzymatic hydrolysis of wood polymers [393] [395] and the biomass destructuration during steam treatment [396]. Hemicellulose and pectin images can be obtained and (acyl) ester modifications inside hemicellulosic components can be quantified as demonstrated by the work of Gou *et al*. [397]. Polarised FTIR microscopy can also give some data concerning the angle of carbohydrates as demonstrated by Chang *et al*. [398] during the deposition of G-layer in *Populus tremula* x *P. alba*, clone INRA 717-1B4. FT-IR microscopy is a non-destructive method to visualise the com-

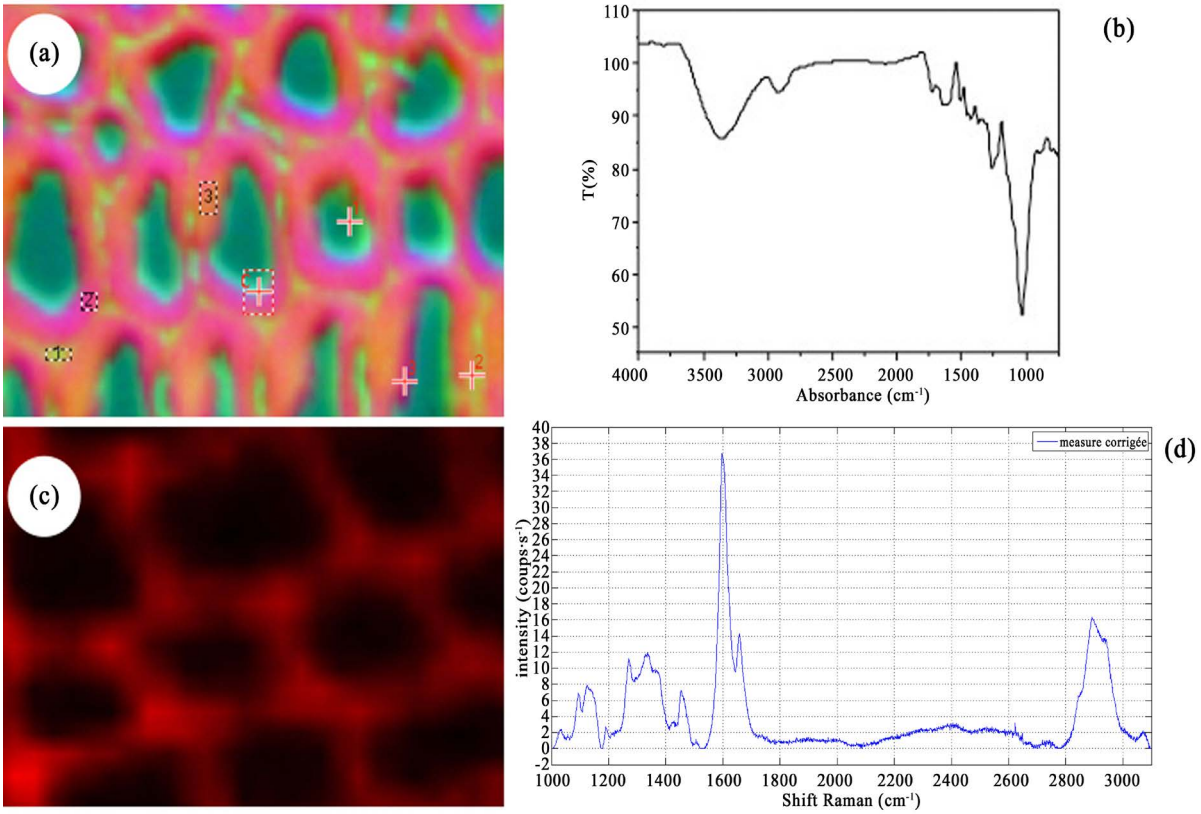

Figure 5. Transvers al section of Douglas-fir softwood. (a) Infrared microscopy picture; (b) infrared spectra extracted from the infrared microscopy picture; (c) Raman microscopy picture obtained from the 1600 cm-1 absorbance band of the spectra ; (d) Raman spectra (Plazanet and Costa, not published).

ponent distribution, which has a resolving power of about one µm sufficient for structural and chemical *in situ* investigation especially for monitoring plant cell wall evolution [399] (**Figure 5(a)**).

Because some chemical functions do not have IR detectable vibrational wavelengths, Confocal Raman Microscopy (CRM) represents a complemenraty approach for non-destructive plant cell wall component analysis [400]. *Arabidospsis* cell walls [401] and spruce wood biomass [400] [402] have been visualised *in situ* with CRM. This technique can be applied to compare wild type and transgenic poplars [403], and normal and compressed wood of pines [404]. As for FTIR microscopy, CRM is a valuable approach to assess the *in situ* effect of biomass modification such as wood degradation for bioraffinery [399] [405] [406]. Limits of this approach are the autofluorescence produced during the laser excitation, and the time necessary for image acquisition. New laser sources and bioinformatic progress are the key to an easier application of this non-destructive method [175] [407]. A considerable advantage of CRM is its resolution level to less than 10 nm (**Figure 5(b)**).

5.6. Mass Spectra Microscopy

Mass spectrometry imaging for plant cell wall analysis can be divided into two groups: TOF-SIMS and MALDI-MS. Secondary ion mass spectrometry (SIMS) exhibits a sub-µm resolution and has first been used for the imaging of elements such as Na^+, K^+, Ca^{2+} in plant cells [408] [409]. Cyro-TOF-SIMS and nano SIMS are two evolutions of SIMS techniques making analysis of high water content materials at lower than ~50 nm level possible [410] [411]. TOF-SIMS has also been used to analyse and conduct direct-mapping of organic compounds [412]. Recently, TOF-SIMS has been applied to investigate wood components, for example lignin and polysaccharides [413] [414] and for monolignol distribution on transversal stem sections of two geniuses of eucalyptus [415]. TOF-SIMS has also been used to investigate heartwood extractable components from *Cryptomeria japonica* such as ferruginol [416] [417]. Because polysaccharides have highly complex glucidic repeat units, MALDI is preferred to SIMS for their identification. Recently, MALDI-TOF MS imaging has been developed

for cellulose and hemicellulose determination on poplar wood transversal section [418].

5.7. NMR Spectroscopy

NMR spectroscopy is a non-invasive and non-destructive technique able to collect information in the native cell wall. Using *in-vivo* solid-state 13C NMR (CP-MAS), Jarvis and Apperley [419] identified cell wall polysaccharides in several seeds and showed their (im)mobility in the wall as well asthe molecular organisationof cellulose. However, the use of CP-MAS NMR leads toa major practical problem, because the application of centrifugal forces can lead to the disintegration of plant tissues. Nevertheless, this technique was used by Fenwick *et al.* [420] to compare the flexibility of polymers in celery collenchyma cell walls.

6. The Future

In vitro and *in situ* methods for plant cell wall polymer characterisation are two complementary approaches. The first approach can accurately describe finesse composition, structure and organisation of cell wall polymers in the 3D network. If polymer extraction has been a useful method applied in the past, some new solvents or some combinational of physical and chemical treatments can become preferable for cell wall polymer disorganisation. This new approach strongly reduces polymer modifications giving the physiologists the opportunity to analyse and understand the interaction between each of them. When polymers are in solution, the complex mixture can then be analysed by immune methods or mass spectrometry. For proteins, mass spectrometry and *de novo* sequencing by MS/MS are the two precedures applied for their identification. For polysaccharides, immune detection is now the fastest method of analysis. High-resolution level of mass spectrometry can represent an alternative to immune detection if polysaccharides are partially hydrolysed with a set of pure enzymes. As tryptic digestion, each polysaccharide of the cell wall can then be partially hydrolysed in oligosaccharides before being identified by MS.

The *in situ* approach probably gives lower quality of results but is suitable to screen a large plant cell wall population for industrial applications. The *in situ* methods are non-destructive and can be carried out after plant biopsy. Here, material preparation and resolution levels of some analytical methods are the limiting factors for their application. For example, tomography instrumentation has recently evolved to high-resolution electro tomography that is a 3D TEM with a resolution at the atomic level [421]. This microscopy has not been applied to the cell wall but to syncytial-type cell plate formation permitting the visualisation of vesicle arrangement at a resolution of ~6nm [422]. In the same order, SIMS are probably the future of *in situ* plant cell wall analysis, but for now the resolution power of the commercial instrumentation does not allow us to access information at the nm level.

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