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Profiling the main cell wall polysaccharides of grapevine leaves using high-throughput and fractionation methods

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ABSTRACT

Vitis species include *Vitis vinifera*, the domesticated grapevine, used for wine and grape agricultural production and considered the world's most important fruit crop. A cell wall preparation, isolated from fully expanded photosynthetically active leaves, was fractionated via chemical and enzymatic reagents; and the various extracts obtained were assayed using high-throughput cell wall profiling tools according to a previously optimized and validated workflow. The bulk of the homogalacturonan-rich pectin present was efficiently extracted using CDTA treatment, whereas over half of the grapevine leaf cell wall consisted of vascular veins, comprised of xylans and cellulose. The main hemicellulose component was found to be xyloglucan and an enzymatic oligosaccharide fingerprinting approach was used to analyze the grapevine leaf xyloglucan fraction. When *Paenibacillus* sp. xyloglucanase was applied the main subunits released were XXFG and XLFG; whereas the less-specific *Trichoderma reesei* EGII was also able to release the XXXG motif as well as other oligomers likely of mannan and xylan origin. This latter enzyme would thus be useful to screen for xyloglucan, xylan and mannan-linked cell wall alterations in laboratory and field grapevine populations. This methodology is well-suited for high-throughput cell wall profiling of grapevine mutant and transgenic plants for investigating the range of biological processes, specifically plant disease studies and plant–pathogen interactions, where the cell wall plays a crucial role.

1. Introduction

Plant polysaccharides constitute some of the most abundant and structurally complex biopolymers found in the natural world (Albersheim, Darvill, Roberts, Sederoff, & Staehelin, 2011). Commonly found in the form of storage polymers (e.g. starch) and structural components (e.g. cell walls) they play a fundamental role in the growth and development of plants. Cell wall polysaccharides are of critical importance in a number of agriculturally and industrially relevant processes; e.g. fruit ripening, plant–pathogen interactions, substrates for biofuels (e.g. wood, coal and oil), paper and pulp industries, and are therefore of considerable economic interest (Albersheim et al., 2011). In relation to agriculture, significant research efforts have been focused on understanding how cell walls mediate the softening and texture properties of fruit during ripening (Albersheim et al., 2011; Cantu, Vicente, Labavitch, Bennett, & Powell, 2008a), and how these polysaccharide components are involved in the dynamic interplay between the host plant and fungal pathogen (e.g. necrotrophs such as *Botrytis cinerea*) in plant disease processes (Albersheim et al., 2011; Cantu et al., 2008b). Considering the importance of cell walls in agricultural research, it is surprising that relatively few crop species have benefited from the tools available to rapidly profile their wall polysaccharides (Nguema-Ona et al., 2012).

Although domesticated grapevine (*Vitis vinifera*) is considered the world's most important fruit crop (OIV, 2009), there is no comprehensive cell wall composition data for grapevine leaves. Weber, Hoesch, & Rast (1995) analyzed leaf-bound phenol components but not carbohydrates) while limited information is available on grape berries (summarized in Moore & Divol, 2011). As no information is available for leaves it is only possible to summarize what

Abbreviations: AIR, alcohol insoluble residue; CoMPP, comprehensive microarray polymer profiling; CBM, carbohydrate binding module; mAb, monoclonal antibody; EPG, endopolygalacturonase; ESI-MS, electrospray ionization-mass spectrometry; FT-IR, Fourier transform-infrared spectroscopy; HPAEC-PAD, high performance anion exchange chromatography-pulse amperometric detection; XEG, xyloglucan-specific endoglucanase; RG, rhamnogalacturonan; HG, homogalacturonan; XyG, xyloglucan; *Psp*XEG5, *Paenibacillus sp* xyloglucan specific endoglucanase 5; *Tr*EGII, *Tricshoderma reesei* endoglucanase II.

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is known regarding grape berries where ripening-related cell wall changes have been surveyed (Moore & Divol, 2011). Earlier studies focused on the changes associated with ripening berries (Davies & Robinson, 2000). Red grapes undergo a change from 'small, green and acidic' berries through a stage termed 'véraison' to 'large, red and sugary' ripe berries just prior to harvest in commercial wine vineyards (Davies & Robinson, 2000). Ripening did not alter the composition of the cellulose and hemicellulosic components (e.g. xyloglucan) to any detectable degree (Nunan, Sims, Bacic, Robinson, & Fincher 1998). However, the pectin components underwent significant alteration (Doco, Vuchot, Cheynier, & Moutounet, 2003; Nunan et al., 1998; Vidal, Williams, Doco, Moutounet, & Pellerin, 2003); particularly the arabinogalactan-II polysaccharides which decreased in abundance (Nunan et al., 1998). A shift in the solubility of the pectin components during ripening appeared to imply a subtle remodelling of the wall (Nunan et al., 1998). Arabinogalactan proteins (AGPs) and rhamnogalacturonan-II (RGII) components of the berry pectin matrix are commonly found in red wine after processing (Doco et al., 2003), whereas the other components are removed (e.g. as skins) or degraded by pectinases (see Arnous & Meyer, 2009 for data on polysaccharide turnover in wine). The importance of cell wall remodelling, biosynthesis and turnover in ripening berries has been further emphasized in recent years through transcriptomic studies (see Goulao, Fernandes, Lopes, & Amâncio, 2012). These studies have highlighted numerous cell wall genes (e.g. expansins, xyloglucanases, pectinases etc.) which correlate with distinct ripening phases (Lücker, Laszczak, Smith, & Lund, 2009; Pilati et al., 2007; Schlosser et al., 2008) indicating the critical contribution polysaccharide organization plays in grape berry development.

Ripening berries are subject to fungal diseases such as grey rot (*B. cinerea*) and mildew (e.g. *Uncinula necator* (powdery mildew) and *Plasmopara viticola* (downey mildew)) which cause significant crop damage worldwide with severe economic consequences for the international grape and wine industries (reviewed in Creasy & Creasy, 2009; Gomès & Coutos-Thévenot, 2009). These pathogenic fungi usually first infect leaves (with younger leaves more vulnerable) damaging vine health and reducing photosynthetic efficiency during the season (Creasy & Creasy, 2009). Thereafter the secondary spread of the fungi to berries occurs, resulting in fruit rot and reducing berry yield/quality. Most preventative control mechanisms are leaf-based, in order to control fungal inoculum sources and overwintering of the pathogen in the permanent structures of the vine. A necrotroph such as *B. cinerea* kills and destroys green tissue while enzymatically degrading the pectin network, whereas a biotroph, such as *Erysiphe necator*, feeds off the living tissue parasitically without causing significant cell death in susceptible hosts (Gomès & Coutos-Thévenot, 2009). Understanding how these fungal diseases progress, given the cell wall is the first barrier to infection and is in effect the battleground (Albersheim et al., 2011), is severely impaired by the lack of information on grapevine leaf cell wall structure and composition. Understanding disease resistance mechanisms, as well as processes relating to leaf ageing and senescence, is dependent on knowledge of the molecular structure and composition of cell walls (Albersheim et al., 2011), conspicuously absent in the scientific literature. Numerous tools are now available to rapidly profile cell wall polysaccharides, including gas liquid chromatography for the determination of cell wall monosaccharide composition (Reiter, Chapple, & Somerville, 1993; Reiter, Chapple, & Somerville, 1997); Fourier transformed infrared spectroscopy (FT-IR; Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000); immunocytcochemistry (Hervé, Marcus, & Knox, 2011; Knox, 1997; Moller et al., 2007); and cell wall degrading enzymes combined with different analytical techniques (Lerouxel et al., 2002; Nguema-Ona et al., 2006; Persson, Sorensen, Moller, Willats, & Pauly, 2011).

This study builds on the profiling approach that was recently optimized and implemented to rapidly analyze the cell wall composition and structure of fully expanded mature tobacco leaves (Nguema-Ona et al., 2012, 2013). The same approach was used here to develop a baseline study of the cell wall polymers of grapevine leaves. A combination of high-throughput techniques were used; including monosaccharide compositional analysis, FT-IR spectroscopy, comprehensive microarray polymer profiling (CoMPP) analysis and oligosaccharide mass fingerprinting as well as more in-depth approaches using chemical and enzymatic fractionation methods were employed. This combination of tools provide a convenient and useful approach to profile the different wall polymers present in developing and mature grapevine leaves. The baseline datasets obtained, and tools evaluated, are essential for screening laboratory plants (for developmental and plant-pathogen studies) and field-grown grapevines (for plant disease monitoring during the season).

2. Experimental

2.1. Plant material

Samples were collected from the Department of Viticulture and Oenology's (Stellenbosch University) experimental vineyards at Welgevallen during October 2010; leaves were collected from a Shiraz vineyard (*V. vinifera* cv. Shiraz) at berry set. Fully expanded photosynthetically active leaves were harvested (samples were taken from five vines and two primary shoots per vine) and flash-frozen using liquid nitrogen and stored at −80 °C until further use. For statistical purposes four biological samples (i.e. leaves) per analyses were utilized with two technical repeats per sample.

2.2. Cell wall isolation and fractionation

Cell wall materials were extracted from frozen leaves and fractionated (using chemical reagents and enzymes) according to the protocol used in Nguema-Ona et al. (2012). Briefly, frozen leaves were ground, under liquid nitrogen using a mortar and pestle, to a fine powder. After successive washes in ethanol, methanol, chloroform the material was de-starched. The fractionation process was performed as described in Nguema-Ona et al. (2012).

2.3. Monosaccharide composition analysis by gas chromatography

A gas liquid chromatography method (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) was used to determine the monosaccharide content of cell wall residues and fractions as described in Nguema-Ona et al. (2012). After hydrolysis (2 M TFA, 110 °C, 2 h), the liberated monosaccharides converted to methoxy sugars using 1 M methanolic HCl at 80 °C for 24 h. Silylation was performed at 80 °C (20 min) to produce trimethyl-silyl-glycosides which were dissolved in cyclohexane. The derivatives were separated and analyzed in a gas chromatograph (Hewlett Packard 5890 series II) coupled to a flame ionization detector, Error bars in the histograms represent the standard deviation (SD) of the mean of four biological samples with two technical replicates per biological sample.

2.4. High performance anion exchange chromatography

Enzyme-generated oligosaccharides were analyzed by high performance anion exchange (HPAE) chromatography Dionex Ultimate 3000 equipped with a CarboPac PA-1 column combined with pulsed amperometric detection (PAD; Coulochem 111 detector). This is as was performed in Nguema-Ona et al. (2012)

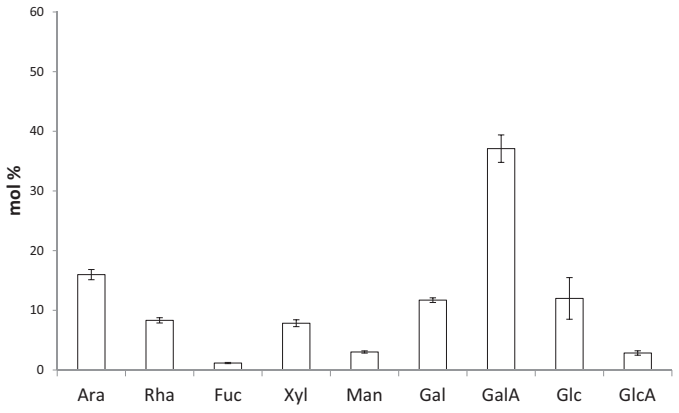

Fig. 1. Monosaccharide compositional analysis (expressed in mol%) of total (de-starched) alcohol insoluble residue prepared from fully expanded grapevine leaves. Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid; GlcA: glucuronic acid.

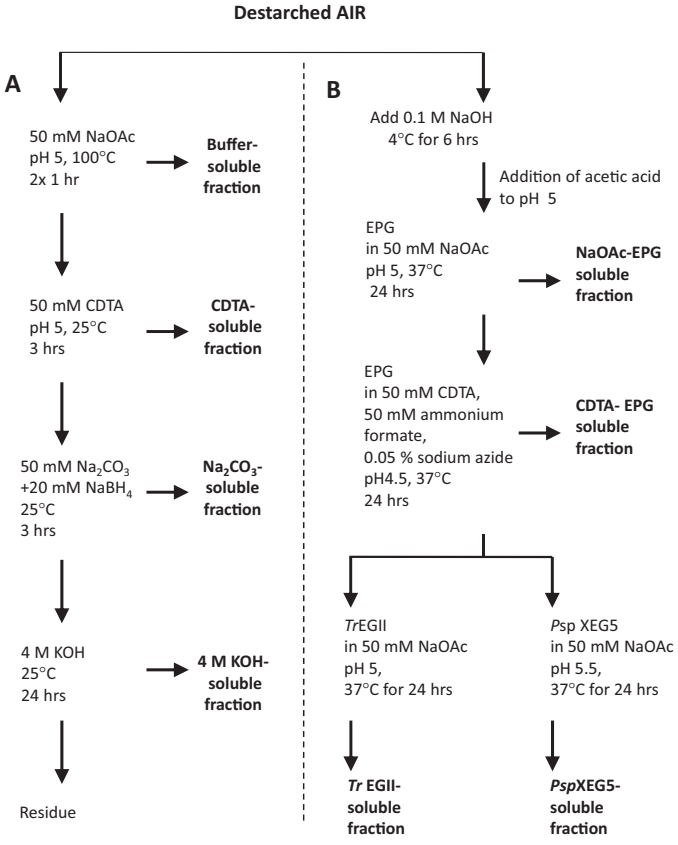

Fig. 2. Flow diagram of the chemical fractionation procedure (A) and enzymatic fractionation procedure (B) performed on alcohol insoluble residue prepared from grapevine leaves. EPG: endopolygalacturonase, *Tr*EGII: *Trichoderma reesei* endoglucanase II, *Psp*XEG5: *Paenibacillus sp* xyloglucan-specific endoglucanase 5.

and the gradient programme used is reported in Nguema-Ona et al. (2013).

2.5. Electrospray ionization-mass spectrometry

The electrospray ionization mass spectrometry (ESI-MS) was performed on a Waters QTOF OPTIMA (Milford, MA, USA). Samples (1 μL) were directly injected into a stream of 80% acetonitrile, 0.1% formic acid using a Waters UPLC at flow rate of 0.2 mL/min. The ion source was set as follows: Source Electrospray positive; Capillary voltage 3 kV; Cone Voltage 40 V. Leucin enkaphalin was used as the lock mass. The mass spectrometer was calibrated with sodium formate.

2.6. Infra-red (IR) spectroscopy of cell wall fractions

A NEXUS 670 FTIR instrument containing a Golden Gate Diamond ATR accessory with a type IIa diamond crystal was used for ATR-FT-IR measurements. The spectra were recorded between 4000 and 650 cm^{-1} with a Geon-KBr beamsplitter and DTGS/Csl detector. Spectral data (128 co-added scans per sample) were processed using Unscrambler™ (Camo® Inc., USA).

2.7. Comprehensive microarray polymer profiling (CoMPP) analysis of cell wall material

AIR was prepared from frozen grapevine leaves as described in the 'cell wall isolation and fractionation' section of this paper. Approximatively 40 mg of material was used to perform the CoMPP analyses as described in Moller et al. (2007). This is as was performed in Nguema-Ona et al. (2012). The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data-set was set to 100 and all other data adjusted accordingly.

3. Results and discussion

Mature grapevine leaf cell walls (Fig. 1) contain a substantial amount of GalA (ca. 35 mol%) with the presence of Rha, Ara and Gal, suggesting that rhamnogalacturonan-I (RG-I), rhamnogalacturonan II (RG-II), and homogalacturonan (HG) are also present. The amount of homogalacturonan is however less than what was found in tobacco (Nguema-Ona et al., 2012), given the lower amount of GalA; ca. 35 mol% in grapevine versus ca. 45 mol% in tobacco. The presence of Xyl and Glc at ca. 10 mol% and 15 mol% respectively, suggest xyloglucans and/or xylans contribute substantially to the composition of non-cellulosic wall polymers in photosynthetically active, fully expanded grapevine leaves.

Chemical and enzymatic fractionation procedures were performed on the grapevine AIR as was conducted on tobacco leaves (see Fig. 2 for a flow-diagram of the protocols followed) (Nguema-Ona et al., 2012). Gravimetric analysis (Fig. 3A) of the chemically fractionated AIR revealed that ca. 8% (w/w) was extracted with hot buffer, ca. 24% (w/w) with the divalent ion chelator 2-diaminocyclohexanetetraacetic acid (CDTA), 6% (w/w) using sodium carbonate treatment, a further 13% (w/w) of material with 4 M KOH while the remaining residue comprised ca. 48% (w/w) of the total AIR.

Monosaccharide compositional analysis was performed to ascertain the main wall polymers present (Fig. 3B–E). The hot buffer soluble material (Fig. 3B) comprised primarily (ca. 55 mol%) of GalA, while the CDTA-extractable fraction (Fig. 3C) showed ca. 75 mol% GalA with some neutral sugars present (Ara, Gal and Rha) indicating the presence of branched pectin (RG-I and RG-II polymers). Combining the data from Fig. 3B and C with the gravimetric data (Fig. 3A), indicates that ca. 30% (w/w) of the total leaf cell wall is pectin. Sodium carbonate extraction produced material composed of ca. 45 mol% GalA, 20 mol% Ara, 10 mol% Rha and 10 mol% Gal (Fig. 3D). Xyl and Glc (at ca. 30 mol% and 20 mol% respectively) were found in the 4 M KOH fraction suggesting xyloglucans and xylans as the main components. Lower levels of Ara (ca. 15 mol%); Gal (ca. 15 mol%), GalA (ca. 15 mol%) and Man (ca. 5 mol%) suggest arabinoxylans and mannans might also be present. The remaining

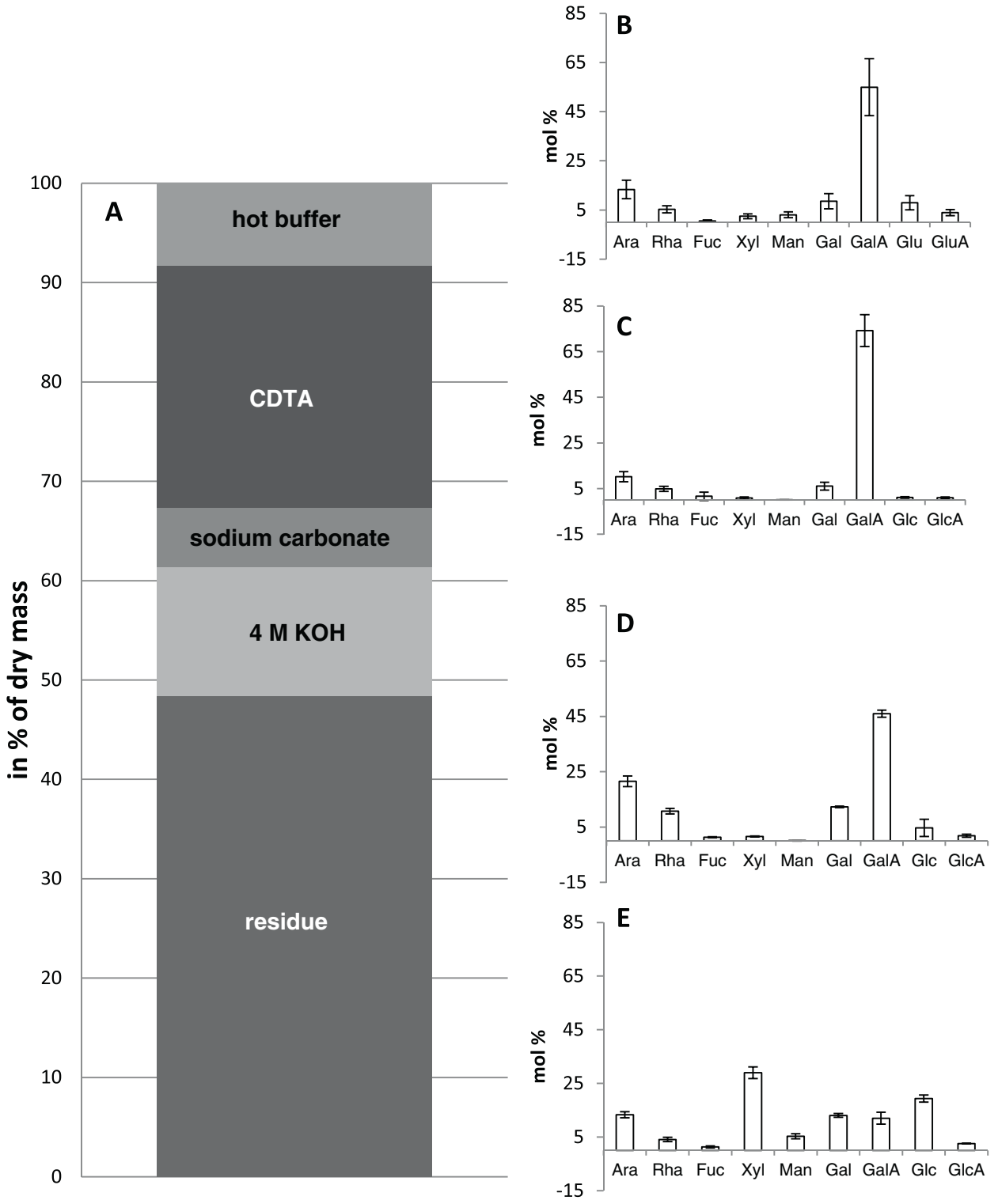

Fig. 3. Gravimetric analysis and monosaccharide composition of various chemically-extracted fractions from alcohol insoluble residue from mature grapevine leaves (for the fractionation process see Fig. 2A). Gravimetric analysis is shown in (A) and monosaccharide composition of the corresponding chemically-extracted fractions in (B–E). (B) 100 °C sodium acetate buffered extract, (C) CDTA extract, (D) Na_2CO_3 extract and E: 4 M KOH soluble extract. Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid; GlcA: glucuronic acid.

residue was found to consist mainly of Glc and some Xyl (data not shown) suggesting crystalline cellulose as the main constituent.

In comparison to the datasets obtained for tobacco (Nguema-Ona et al., 2012) the grapevine gravimetric data deviates in two major ways; (1) the insoluble residue comprises ca. 50% of the grapevine AIR in comparison to only ca. 15% in the tobacco samples, and (2) the bulk of the pectin in grapevine AIR required CDTA to effect extraction whereas hot buffer was sufficient for tobacco

leaves. Concerning the insoluble residue in grapevine leaves this abundance is very likely due to the major leaf veins present, which comprise mainly xylans and cellulose; such extensive reinforced vascular networks are not present in the pectin-rich tobacco leaves. While the CDTA data for grapevine is an indicator that calcium cross-linking is critical for pectin integrity in this species, this appears not to be the case for tobacco. This data is particularly interesting in relation to plant–pathogen interactions for grapevine where *B. cinerea* facilitates its degradation of leaves and fruit by secreting organic acids (e.g. oxalic acid; a calcium chelator) and ePGs which target pectin networks.

Fourier-transform infrared (FT-IR) spectroscopy was used to provide further support for the presence of specific wall polymers in the different fractions (Fig. 4). Inspection of spectra of total AIR revealed maxima at 1580 cm^{-1}, 1041 cm^{-1} and at 1017 cm^{-1}, as well as bands between 1200 and 1300 cm^{-1} (Fig. 4A). This grapevine AIR spectrum is very different from the tobacco data previously reported on (Nguema-Ona et al., 2012). Although pectin is present in substantial amounts (from the 1017 cm^{-1} peak indicating HG presence) there is also ample hemicellulose polymers as evidenced by the broad shoulders, including peaks at 1041 cm^{-1}, and protein peaks at 1580 cm^{-1} (Kacurakova et al., 2000). Chemically fractionated preparations of AIR (Fig. 2A) were probed using FT-IR spectroscopy to investigate the pectic and hemicellulosic polymers of the wall network (Fig. 4B–E). The hot buffer fraction displayed a major maxima at 1017 cm^{-1} (HG chains) and local maxima at 1580 cm^{-1} (amides) and 1740 cm^{-1} (esters) (Fig. 4B) (Kacurakova et al., 2000). CDTA and sodium carbonate extracted material showing major absorbance bands at 1580 cm^{-1} and 1380 cm^{-1} (Fig. 4C and D). Residual CDTA in the fractions were responsible for the bands, however as this region is not in the carbohydrate region it did not influence data analysis (Fig. 3A), although probably contributes to an overestimation in the gravimetric analysis. The CDTA and sodium carbonate spectra (Fig. 4C and D) show some absorbance in the pectin-rich region 1200–900 cm^{-1}, but the peak at 1041 cm^{-1} would suggest hemicelluloses present. The presence of a 1041 cm^{-1} peak in the KOH fraction would suggest XyG absorption, as well as protein (1640 cm^{-1}) (Kacurakova et al., 2000) (Fig. 4E). The remaining residue was found to be mainly cellulose (data not shown).

CoMPP analysis of grapevine AIR was performed as outlined in Nguema-Ona et al. (2012) except cadoxen was omitted. CoMPP analysis uses sets of monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) to profile plant cell wall-glycan epitopes (Moller et al., 2007). CoMPP analysis provides information on polysaccharide rather than monosaccharide occurrence (Moller et al., 2007). The CoMPP data (Fig. 5) strongly supported the chemical and spectroscopic data reported. The CDTA extract should be similar to the combination of the hot buffer and CDTA extracts performed for gravimetric analysis. The NaOH extracted material probably represents a combination of the sodium carbonate and KOH fractions. As the CoMPP analysis does not directly compare with the fractionation scheme followed for the gravimetric analyses it is not possible to be more precise. Analysis of the CDTA extract (Fig. 5) showed that the HG epitopes recognized by mAbs JIM5, JIM7, LM18, LM19 and LM20 were present at high relative abundance and confirmed that HG polymers predominated in the AIR. The binding of mAbs LM5, LM6 and LM13 (Fig. 5), indicated the presence of galactan and arabinan epitopes, consistent with branched pectin RG-I domains. Additionally, arabinogalactan proteins (AGPs) appeared also to be extracted by the CDTA extract, as shown by the binding of mAbs JIM8 and JIM13, and to a lesser extent LM2 (Fig. 5). Some extensins (mAb JIM20) and xyloglucans (mAb LM15) were also extractable with CDTA (Fig. 5). The binding of mAb LM15, at high relative intensity,

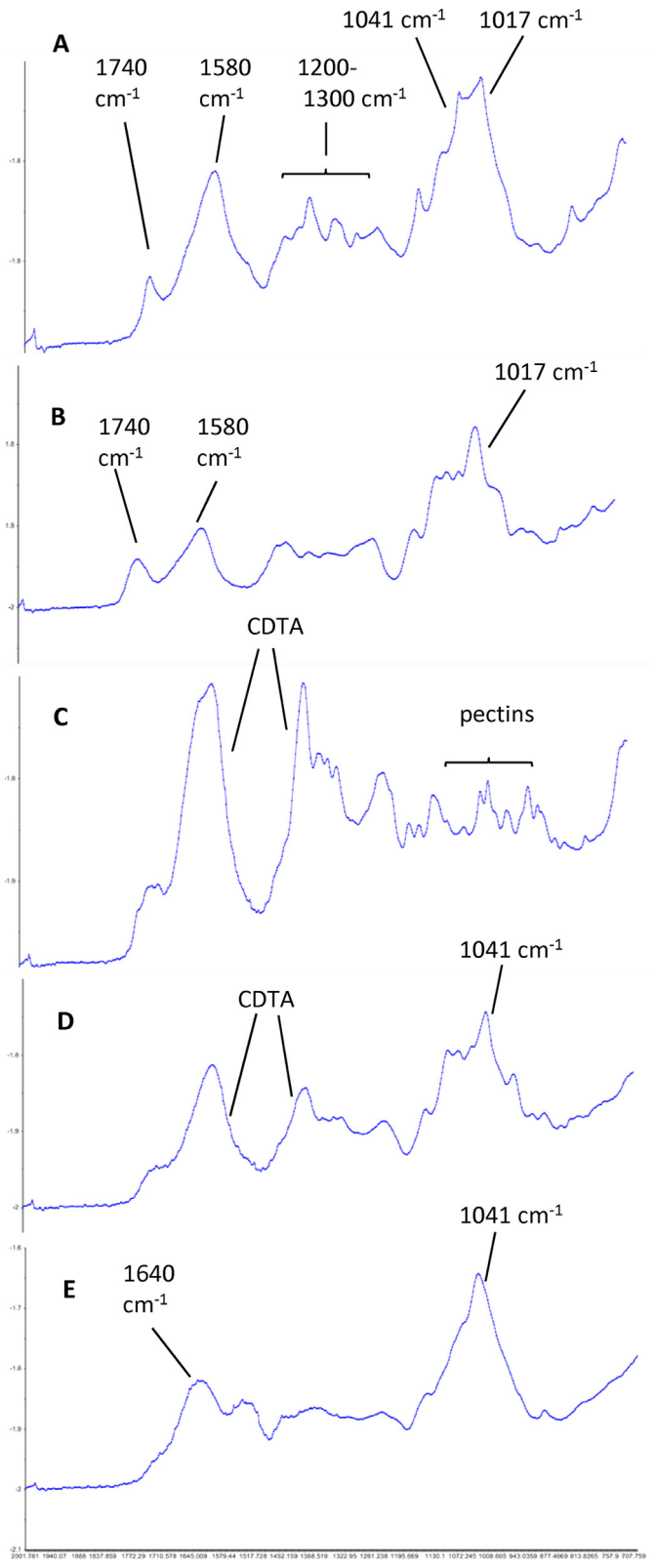

Fig. 4. Fourier transform infrared (FT-IR) spectra generated from alcohol insoluble residue prepared from grapevine leaves and associated chemical fractions (for the fractionation process see Fig. 2A). (A) total alcohol insoluble residue, (B) 100 °C sodium acetate buffered extract, (C) CDTA extract, (D) Na_2CO_3 extract and (E) 4 M KOH extract.

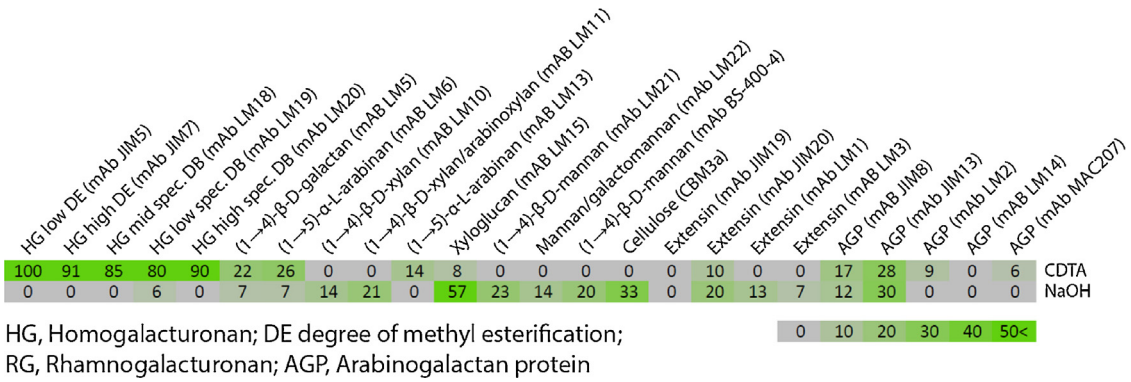

Fig. 5. CoMPP (comprehensive microarray polymer profiling) analysis of grapevine leaf cell wall fractions. The heatmap shows the relative abundance of plant cell-wall glycan-associated epitopes present in grapevine leaf cell wall material and colour intensity is correlated to mean spot signals. Sequential extractions were carried out with CDTA (top row) and NaOH (bottom row); and the extracted material spotted onto nitrocellulose which was probed with sets of antibodies and carbohydrate binding modules. The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data set was set to 100 and all other data adjusted accordingly. A cut off value of 5 was imposed.

to the NaOH fraction (Fig. 5) supported a XyG-rich composition (Fig. 3E). Additional epitopes recognized as xylans (mAb LM10), arabinoxylans (mAb LM11), mannans (mAbs LM21 and BS-400-2) and galactomannans (mAb LM22) were present (Fig. 5). The NaOH extract showed binding by anti-(1 → 3)-β-glucan mAb BS-400-4 and anti-cellulose CBM3a, suggesting that the extract was rich in hemicellulosic/cellulosic polymers (Fig. 5). Co-extracted pectic- and AGP-containing material were also present, indicated by the binding of mAbs LM5, LM6, JIM8 and JIM13 to the NaOH extracted material (Fig. 5). Extensins are also extracted as evidenced by the binding of mAbs JIM20, LM1 and LM3 (Fig. 5).

In addition to the chemical fractionation, enzymatic fractionation was also performed (see Fig. 2B) to 'dissect' the wall polymer networks (Persson et al., 2011). This involved digestion with endopolygalacturonase (EPG)-containing preparations, and two different XEG enzymes (Fig. 2B). Monosaccharide compositional analysis of the fractions obtained from EPG digestion in buffered sodium acetate yielded GalA (ca. 35 mol%), Gal (ca. 15 mol%), Ara (ca. 15 mol%), Glc (ca. 12 mol%) and Rha (ca. 8 mol%) (Fig. 6A). This again suggested a pectin-rich extract composed of both HG chains and branched structures (e.g. RG-I). Further treatment of the residue from the first digestion with EPG supplemented with CDTA resulted in the release of additional pectic material (Fig. 6B) as evidenced by the presence of GalA (ca. 60 mol%), Gal (ca. 8 mol%), Ara (ca. 15 mol%), Glc (ca. 8 mol%) and Rha (ca. 8 mol%). FT-IR spectroscopy of both EPG liberated fractions confirmed the pectic nature with strong absorbance maxima in both spectra at 1017 cm^{-1} (data not shown).

The de-pectinated residue produced after EPG and CDTA-EPG treatment was used to extract and analyze the XyG structure of grapevine leaves. The *Tr*EGII and *Psp*XEG5 were described and compared with respect to specificity-of-action (see Nguema-Ona et al., 2012). Digestion of the de-pectinated with the *Tr*EGII yielded Xyl and Glc at ca. 26 mol% and 32 mol% respectively (Fig. 6C). These data confirm the XyG-rich nature of the extract, however high amounts of Xyl and Man (Fig. 6C) also indicate xylans and mannans are present. As this endoglucanase preparation is known to possess glycanase activity (www.megazyme.com) this is unsurprising. In contrast, use of the *Psp*XEG5 preparation (see Fig. 2B) releases Glc (ca. 55 mol%), Xyl (ca. 25 mol%), Gal (ca. 10 mol%) and GalA (ca. 5 mol%) as the major sugars (Fig. 6D). The ca. 2:5 molar ratio for Xyl:Glc indicates the dominant xyloglucan component, however Gal, GalA, Ara and Rha suggest additional pectin co-extracted.

HPAEC and ESI-MS analyses were performed on oligomeric products of the two enzyme treatments. The analyses were carried out on de-pectinated AIR residue which is enriched for xyloglucan components. HPAEC analysis of the *Tr*EGII extract yielded three major (two peaks co-elute) (Fig. 7A) whereas *Psp*XEG5 digestion produced only two oligosaccharides (Fig. 7B). ESI-MS analysis provided further structural information on the components in both *Tr*EGII and *Psp*XEG5 extracts (Fig. 7C and D). The main ions present in both digests (Fig. 7C and D) at m/z 1085, 1247, 1394 and 1555 correlated to Hex_4Pen_3, oligomers respectively. The only major difference observed between ESI-MS spectra of *Tr*EGII- versus *Psp*XEG5-mediated digestion (Fig. 7C and D) was the conspicuously low abundance of m/z 1085 in the *Psp*XEG5-mediated digestion (Fig. 7D) versus *Tr*EGII-mediated digestion (Fig. 7C).

More detailed information was obtained by employing fractionation techniques, using chemical and enzymatic tools, to sequentially degrade and reveal the major sub-networks, pectin and hemicellulose, present in the leaf AIR. This is further emphasized in the enzymatic fractionation data for grapevine AIR where EPG alone did not liberate much HG-rich pectin whereas inclusion of CDTA with EPG addition extracts substantially higher levels of HG-rich material. Further analysis of the XyG components (exposed in the de-pectinated samples) with *Psp*XEG5 enzyme produced XXFG and XLFG subunits (Fry et al., 1993) which are typical of dicotyledonous plants; however the *Tr*EGII enzyme was more non-specific liberating the XXXG motif (Fry et al., 1993) and other hemicelluloses (e.g. xylan and mannan oligomers) also present. Clearly, as suggested for tobacco leaves (Nguema-Ona et al., 2012), the *Tr*EGII enzyme would be much better for screening grapevine populations since it would assay variation in XyG, mannans and xylans simultaneously.

Each of the profiling methods; (1) high-throughput screening using GC-based monosaccharide composition, FT-IR spectra and CoMPP datasets and, (2) fractionation methods (e.g. gravimetric analysis) coupled to HPAEC and mass spectrometric tools, provide complementary sets of information. A comparison of the grapevine data (this study) with similar datasets generated for *Arabidopsis thaliana* (Lerouxel et al., 2002; Moller et al., 2007; Zablackis, Huang, Müller, Darvill, & Albersheim, 1995) and *N. tabacum* (Nguema-Ona et al., 2012) revealed marked differences in the profiles obtained. The limited molecular genetic resource (e.g. mutants and transgenic lines) available for grapevine unfortunately prevents a thorough validation of the cell wall data, as was performed for *A. thaliana*.

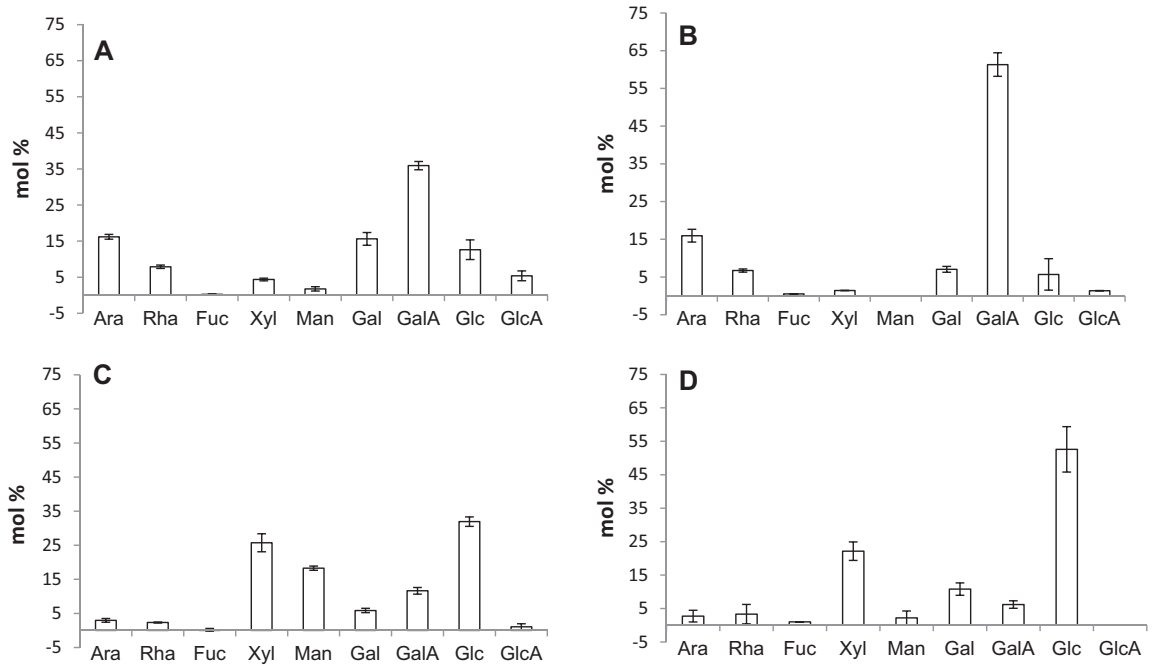

Fig. 6. Monosaccharide compositional analysis of EPG-mediated and XEG-mediated soluble fractions of alcohol insoluble residue prepared from grapevine leaves (for the fractionation process see Fig. 2B). (A) Fraction resulting after EPG digestion of previously saponified alcohol insoluble residue (NaOAc-EPG soluble fraction); (B) Fraction resulting after EPG digestion in the presence of a chelating agent (CDTA) of the residue (CDTA-EPG soluble fraction); (C) Fraction resulting after *Tr*EGII digestion of de-pectinated residue produced after EPG and CDTA-EPG treatment; (D) Fraction resulting after *Psp*XEG5 digestion of the de-pectinated residue produced after EPG and CDTA-EPG treatment.

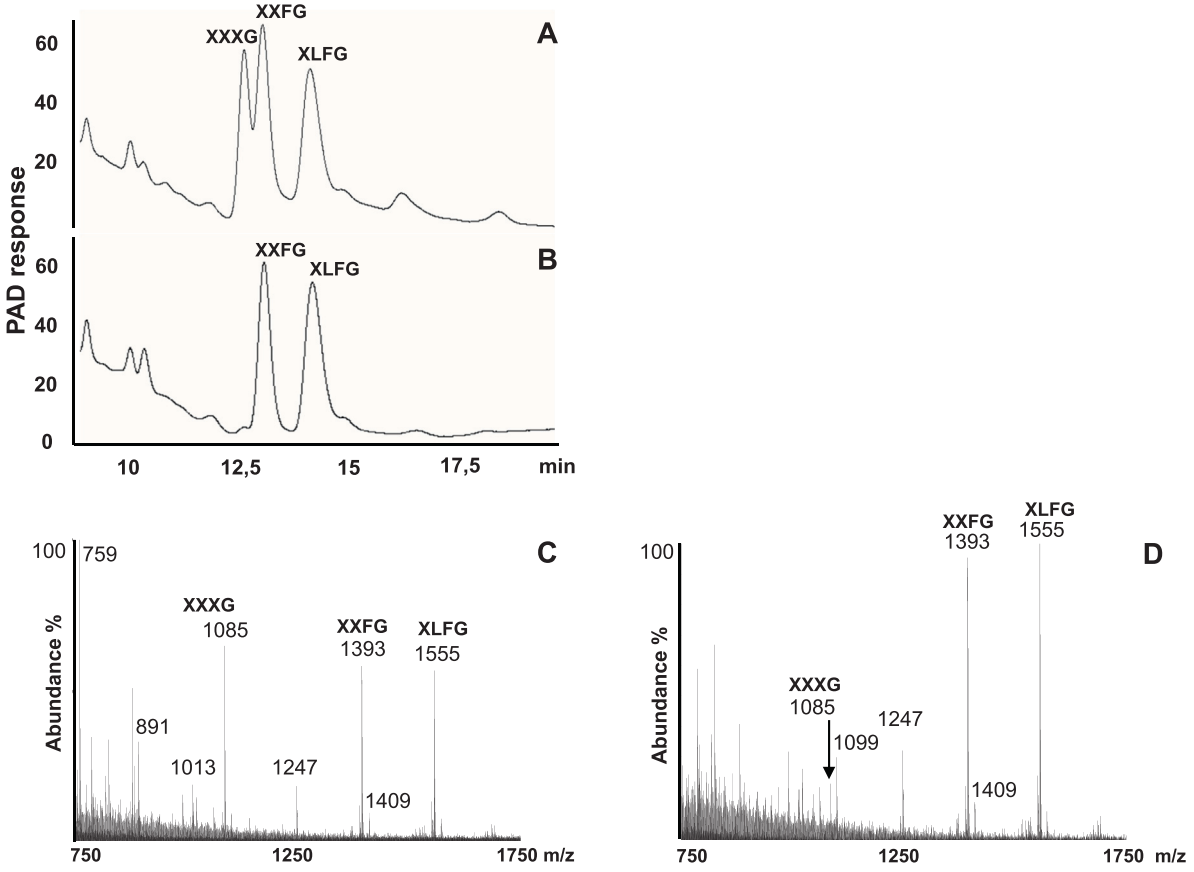

Fig. 7. A comparison of oligosaccharides obtained from grapevine xyloglucans (enriched from de-pectinated alcohol insoluble residue) using *Trichoderma reesei* endoglucanase II (*Tr*EGII) or *Paenibacillus sp* XEG5 (*Psp*XEG5) enzyme treatment followed by (a) high performance anion exchange chromatography (A *Tr*EGII, B *Psp*XEG5) and (b) ESI mass spectrometry (C *Tr*EGII, D *Psp*XEG5).

4. Conclusion

Grapevine is the major international agricultural fruit tree crop with significant land areas under vine cultivation in Europe (e.g. Spain, France and Italy), North America (e.g. the USA), South America (e.g. Chile and Argentina), South Africa, Australia and the East Asian region, particularly India and China (OIV, 2009). Consequently there is a significant need for the development and optimization of new screening tools to analyze grapevine plants, including their cell wall properties. Disease remains a significant factor in reducing photosynthetic yield (e.g. leaf damage), sugar loading and berry quality in cultivated vineyards with severe economic consequences for grape and wine industries around the globe. Fungal diseases, such as grey rot (*B. cinerea*) and mildew (*E. necator*), are a significant problem in vineyards of all major international grape and wine producing countries. Profiling field-grown and model laboratory vine plants are necessary to provide baseline datasets for anti-fungal research on grapevine pathogens and functional gene studies. The lack of suitably validated cell wall profile methods and reference datasets on mature grapevine leaf tissue is a substantial barrier to progress in studying field-grown non-transgenic and laboratory transgenic *V. vinifera* populations generated for scientific study. Although previous studies have been conducted on grapevine tissue and cell wall characterization, these studies were generally focused on berries, employed classical chemical polysaccharide isolation methods and/or were restricted to specific components/systems (e.g. phenolic components, suspension culture systems). Thus a significant gap existed with respect to the leaf cell wall profile which has now been remedied by this study.

But more importantly state-of-the-art methods optimized on the model plant, *A. thaliana*, and recently on *N. tabacum* (Nguema-Ona et al., 2012) have now been extended to grapevine. This set of techniques can form part of a growing suite of molecular-analytical methods to profile vineyards and transgenic laboratory populations. The major differences found between tobacco and grapevine are: (1) the presence of significant vascular veins which correlate with the significant insoluble residue found in grapevine material after fractionation, (2) the significant proportion of chelator soluble pectin present in grapevine AIR suggesting calcium cross-linking as being very important, and (3) the standard XXXG, XXFG, and XLFG fucosylated xyloglucan in contrast to the arabinoxyloglucan present in tobacco leaves (see Nguema-Ona et al., 2012). As found for tobacco (Nguema-Ona et al., 2012, 2013), only a general impression of the cell wall is obtained from monosaccharide composition and FT-IR spectra alone, whereas the CoMPP analysis provides a much richer and detailed dataset on polymer occurrence, via epitope frequency and binding intensity. Combining all three datasets, coupled with chemometric data analysis methods, allows for a particularly powerful screening approach to evaluate field and laboratory populations.

The unique wall profile obtained for *V. vinifera* emphasizes the necessity for producing baseline datasets for all important model plants, as was performed previously for *N. tabacum* (Nguema-Ona et al., 2012) and now for grapevine. This profiling approach is relevant for other dicotyledonous crops and is now an invaluable addition to the molecular toolbox of scientists working in the international grapevine research community.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.013.

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