Bioenerg. Res. (2016) 9:969–979
DOI 10.1007/s12155-016-9770-y

# *Eucalyptus* Cell Wall Architecture: Clues for Lignocellulosic Biomass Deconstruction

**Marcela Mendes Salazar[1,2] · Adriana Grandis[3] · Sivakumar Pattathil[4] · Jorge Lepikson Neto[1,2] · Eduardo Leal Oliveira Camargo[1] · Ana Alves[5] · José Carlos Rodrigues[5] · Fabio Squina [6] · João Paulo Franco Cairo[1,6] · Marcos S. Buckeridge[3] · Michael G. Hahn[4] · Gonçalo Amarante Guimarães Pereira[1]**

Published online: 14 July 2016


**Abstract** The architecture, composition, and chemical properties of wood cell walls have a direct influence on the process that occurs prior to fermentation in second-generation biofuel production. The understanding of the construction patterns of cell wall types is the key to the new era of second-generation biofuels. *Eucalyptus* species are great candidates for this purpose since these species are among the fastest growing hardwood trees in the world and they have been improved for biomass production. We applied the glycome profiling and other combined techniques to study xylem cell walls of three economically important species (*Eucalyptus globulus*, *Eucalyptus grandis*, and *Eucalyptus urophylla*). Glycome profiling analyses revealed that species differ in the same key aspects of cell wall polymer linkages, with *E. globulus* and *E. urophylla* presenting contrasting phenotypes, and *E. grandis* with intermediate characteristics. *E. urophylla* is known for high recalcitrance, that is probably determined by the strong associations between lignin and cell wall polymers, and also lignin content. On the other hand, *E. globulus* cell wall polymers are loosely linked, so its cell wall can be easily deconstructed. We have shown in this work that the composition of cell walls differs in quantity and quality among the *Eucalyptus* species and such variations in composition influence the process of lignocellulosic feedstock assessment. However, the greatest influence relies on the amount and type of associations between cell wall polymers. A high yield of cellulose, from any biomass source, directly depends on the cell wall architecture.

**Keywords** Bioethanol · Cell wall architecture · Glycome profiling · *Eucalyptus* · Wood

**Abbreviations**

| | |
|---|---|
| 1 M | 1 Molar |
| 4 M | 4 Molar |
| AIR | Alcohol-insoluble residues |
| DMSO | Dimethyl sulfoxide |
| Ara | Arabinose |
| cP/cH | Pentose/hexose |
| Fuc-XG | Fucosylated xyloglucan |
| GalA | Galacturonic acid |
| Glc | Glucose |
| Gal | Galactose |
| HG | Homogalacturonan |
| H/G | Hydroxyphenyl/guaiacyl |
| LCC | Lignin-carbohydrate complex |
| Man | Mannose |
| S/G | Syringyl/guaiacyl |
| Non-fuc XG | Non-fucosylated xyloglucan |
| PC | Post chlorite |

✉ Gonçalo Amarante Guimarães Pereira
goncalo@unicamp.br

[1] Genomic and Expression Laboratory, Department of Evolution Genetics and Bioagents, Institute of Biology, University of Campinas, Campinas, Brazil

[2] Senai Innovation Institute for Biomass, Três Lagoas, Brazil

[3] Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, Brazil

[4] BioEnergy Science Center, Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA

[5] Tropical Research Institute of Portugal (IICT), Forestry and Forest Products Group, Lisbon, Portugal

[6] Brazilian Bioethanol Science and Technology Laboratory (CTBE), Campinas, SP, Brazil

| | |
|---|---|
| RG | Rhamnogalacturonan backbone |
| RG/AG | Rhamnogalacturonan/arabinogalactan |
| RG-I | Rhamnogalacturonan I |
| RG-II | Rhamnogalacturonan II |
| Rha | Rhamnose |
| XG | Xyloglucan |
| Xyl | Xylose |
| Fuc | Fucose |

## Introduction

Global demand for sustainable alternatives to fossil fuels has increased research efforts towards identifying new sources for biofuels [1–3]. Woody feedstock is of great interests as it stores a large amount of fermentable sugars in its structure [4, 5]. In wood, cellulose, hemicellulose, and lignin comprise the main chemical components present in secondary cell walls [6, 7]. These compounds, together with hemicelluloses, pectins, and proteins are chemically linked forming a complex structure [8, 9]. The intricate arrangement of the cell walls causes biomass recalcitrance, which hampers the conversion of cellulose into fermentable sugars. Consequently, this recalcitrant material requires pre-treatments to facilitate the depolymerization of the wall, thus becoming a challenge because of the decreased cost-effectiveness of the whole process [10–13].

*Eucalyptus* species are among the fastest growing hardwood trees in the world [14, 15]. They have been grown mainly for pulp and paper industries, with commercial plantations reaching over 90 countries [16, 17]. Their favorable characteristics for wood production (fast growth, high yield, good response to cultivation and breeding management) made the genus an important feedstock in the world's forestry sector [18]. All these characteristics are also favorable to second-generation biofuel production [15].

In Brazil, three *Eucalyptus* species (*Eucalyptus globulus*, *Eucalyptus grandis*, and *Eucalyptus urophylla)* with different wood quality characteristics are widely used in breeding programs [18]. Regarding the cellulose productivity, these species are from best to worst: *E. globulus*, *E. grandis*, and *E. urophylla*, with *E. globulus* presenting the most desirable wood properties for cellulose yields and *E. urophylla* with higher recalcitrance for cellulose assessment. However, in terms of adaptability to biotic and abiotic stress, they are in the opposite order [14, 16, 19, 20]. Conventional genetic improvement programs have moved forward on seeking trees that produce more cellulose and have lower lignin content. However, not only for *Eucalyptus* species but also biomass recalcitrance is still a challenge for second-generation biofuel technologies [21, 22].

The composition, structure, and chemical properties of cell walls directly influence the fermentable sugar extractability [23–26]. In this context, our goal was to understand the difference in composition and structure of cell walls and may determine their deconstruction patterns.

In the present work, we applied glycome profiling and other combined techniques (liquid chromatography for monosaccharide analysis, enzymatic hydrolysis, and lignin determination) to study the xylem cell walls of three economically important species. Glycome profiling uses a comprehensive suite of monoclonal antibodies that recognize most major non-cellulosic wall polysaccharides, enabling us to characterize and monitor the cell wall structure and extractability of its components [27, 28]. We found that different species display distinct patterns of cell wall structure and that this architecture may directly affect cellulose access of these materials. To our knowledge, this is the first report of *Eucalyptus* cell wall fine structure and comparison between three economically important species, critical information that could lead the development of better parameters for pre-treatment and hydrolysis procedures for biofuel production.

## Results

### Analyses of the Cell Wall Non-cellulosic Polysaccharides

Isolated cell walls were submitted to sequential extractions with chemicals with increasing harshness [28]. Each fraction were named after the chemicals used in extraction as follows: ammonium oxalate (named ammonium), sodium carbonate (carbonate), 1 M KOH (1 M), 4 M KOH (4 M), sodium chlorite (chlorite), and 4 M KOH post chlorite (4 M PC). ELISA tests with 155 monoclonal antibodies were performed for each sample and extract. The toolkit of antibodies recognizes most non-cellulosic polysaccharides in the wall. For each cell wall fraction, the monosaccharide yields were also determined.

#### *Glycome Profiling*

To provide more detailed information on cell wall compositional and structural differences between young *Eucalyptus* trees, we obtained the glycome profiling for three *Eucalyptus* species (young trees): *E. globulus*, *E. grandis*, and *E. urophylla* (Fig. 1). Table 1 presents the statistical analysis of the different composition in cell wall extracts (*p* value) through the comparison between species of each group of carbohydrate epitopes in Fig. 1.

The glycome profiling of *E. globulus* and *E. grandis* showed a similar pattern of extractability and abundance of cell wall glycan epitopes, with some specific differences discussed below. However, the overall pattern of extractability and abundance of cell wall glycan epitopes for *E. urophylla* was different from the other two species, which became pronounced in the last two most harsh extracts (chlorite and 4 M PC). The differences found are potentially crucial in determining the final pattern of the cell wall architecture of the different species.

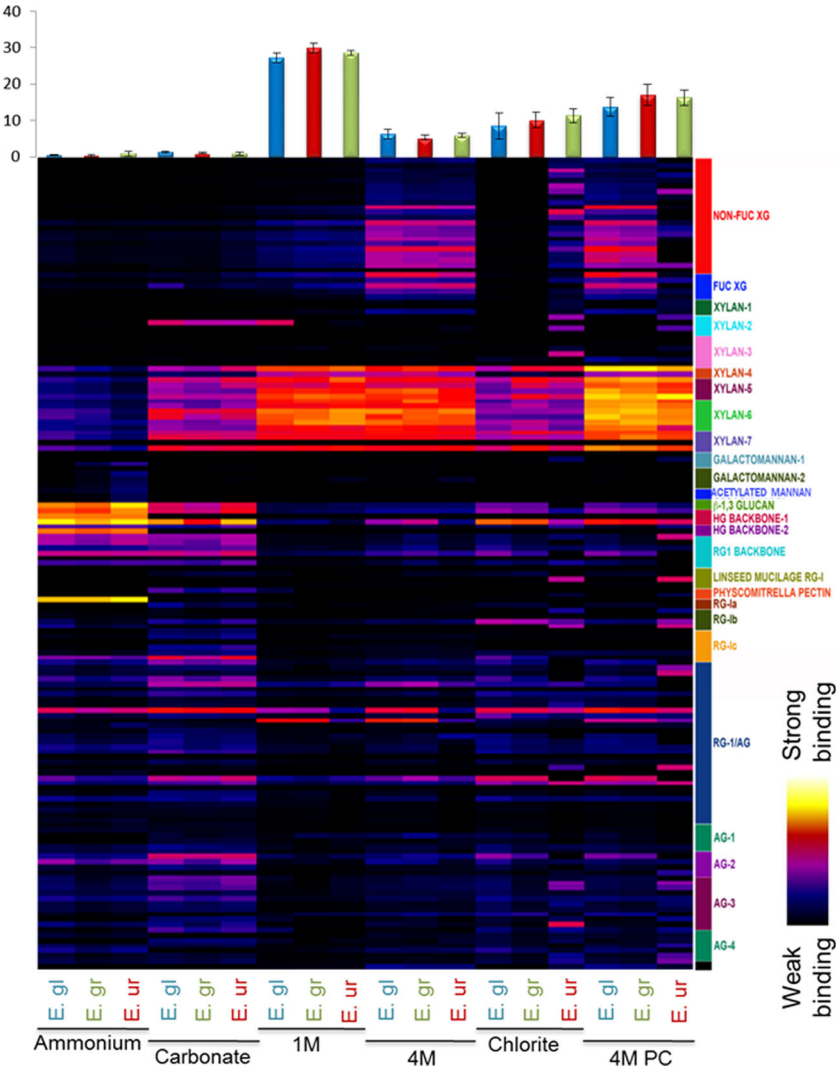


**Fig. 1** Glycome profiling of xylem of *Eucalyptus* species. Sequential extracts of alcohol-insoluble residues (AIR) of each species are presented (as labeled at the bottom: ammonium: ammonium oxalate; carbonate: sodium carbonate; 1 M: 1 M KOH; 4 M: 4 M KOH; chlorite: sodium chlorite; 4 M PC: 4 M KOH post chlorite). The *legend panel on the right* displays the identity of the polysaccharides predominantly recognized by each group of mAbs. Antibody binding is represented as colored heat maps, as showed by the pallet key. The bar graphs at the top indicate the amount (mg) of soluble material recovered at each extraction step per gram of AIR

Although the total amount of carbohydrates extracted in the ammonium and carbonate extracts are low, this extracts are rich in pectin, as indicated by the higher abundance of pectic epitopes recognized by homogalacturonan (HG), rhamnogalacturonan backbone (RG) and rhamnogalacturonan/arabinogalactan (RG/AG) groups of antibodies. These epitopes are also observed in all sequential extracts, despite being stronger in ammonium and carbonate extracts. Pectic epitopes recognized by the RG/AG and AG groups showed differential abundance between the species in most extracts, as shown in Table 1.

The abundance of galactomannan epitopes was observed to be in trace for all species and extracts. However, we have found that, in ammonium and carbonate extract, galactomannan epitopes were differently abundant between species (stronger in *E. grandis* and *E. urophylla*) (Fig. 1 and Table 1).

The presence of xylan epitopes recognized by the xylan group of antibodies was observed in all extracts, becoming remarkably abundant with KOH extracts. Also, in the ammonium extract, a lower abundance was observed in *E. urophylla*. Xyloglucan (XG) epitopes recognized by the non-fucosylated XG (Non-fuc XG) and Fuc-XG group of antibodies are present in higher abundance in the 4 M KOH extracts for all species, with some differences between species as shown in Table 1.

The most remarkable difference was found in the abundance of all *E. urophylla* epitopes related to the other species after the chlorite treatment (which removes mainly lignin). In the chlorite extract, there is a considerable abundance of xylan epitopes recognized by specific xylan antibodies (from groups 1 to 3) and XG epitopes only in *E. urophylla*, while in the 4 M PC extract the abundance of these epitopes is lower in *E. urophylla.* Also, pectic epitopes recognized by the RG/AG showed statistically difference in abundance in *E. urophylla* (Table 1).

### *Determination of Monosaccharides*

The monosaccharide yields were determined using the same cell wall extracts used in the glycome profiling (Table 2). The general composition of *Eucalyptus* xylem cell wall was similar with some specific differences.

**Table 1** Glycome profiling statistics

| | Ammonium | | Carbonate | | 1 M | | 4 M | | Chlorite | | 4 M PC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E.gl/E.gr/E.ur | *p* value | E.gl/E.gr/E.ur | *p* value | E.gl/E.gr/E.ur | *p* value | E.gl/E.gr/E.ur | *p* value | E.gl/E.gr/E.ur | *p* value | E.gl/E.gr/Eur | *p* value |
| Non-fuc | a/a/a | 0.424 | a/a/B | 0.00 | a/a/a | 0.24 | a/a/a | 0.423 | a/a/B | 0.00 | A/A/b | 0.00 |
| Fuc-Xyl | a/a/a | 0.294 | a/a/a | 0.268 | a/a/a | 0.136 | a/a/a | 0.736 | a/a/B | 0.00 | A/A/b | 0.00 |
| Xylan | A/A/b | 0.001 | a/a/a | 0.696 | a/a/a | 0.978 | a/a/a | 0.96 | a/a/a | 0.111 | a/a/a | 0.959 |
| Galctom | a/B/B | 0.001 | a/a/B | 0.00 | a/a/a | 0.061 | a/a/a | 0.459 | a/a/a | 0.065 | a/a/a | 0.137 |
| HGbk | a/a/a | 0.084 | a/a/a | 0.792 | a/a/a | 0.882 | a/a/a | 0.464 | a/a/a | 0.166 | a/a/a | 0.679 |
| RG | a/a/a | 0.728 | a/a/a | 0.426 | a/a/a | 0.249 | a/a/a | 0.965 | a/a/a | 0.823 | a/a/a | 0.026 |
| RG/AG | A/Ab/b | 0 | a/a/a | 0.199 | aB/a/B | 0.012 | a/a/a | 0.066 | A/b/b | 0.00 | aB/a/B | 0.099 |
| AG | a/a/a | 0.068 | a/a/a | 0.578 | a/a/a | 0.29 | Ab/A/b | 0.027 | a/a/a | 0.172 | a/a/a | 0.254 |

*p* value of glycome profiling between species in each extract presented in Fig. 1. The comparison was performed for ELISA results added for most carbohydrate groups recognized for monoclonal antibodies. Different letters indicate significant differences between species ($p < 0.05$) by ANOVA test or Kruskal-Wallis test. Capital and lowercase letters indicate more and less content of the carbohydrate respectively

Xylose (Xyl) and fucose (Fuc) were observed as being the higher and lesser abundant monosaccharides in all extracts, respectively, except in the ammonium extract where glucose (Glc) is more abundant than Xyl. Moreover, the 1 M extract was almost exclusively composed of Xyl (Table 2).

In the ammonium extract, Glc was the most abundant monosaccharide (~58.8 %), very likely associated with starch and not cell wall. The cell wall components in this fraction are Xyl (~23 %) and those likely associated with the pectin monosaccharides (galactose—Gal ~7.9 %, arabinose—Ara ~3.51 %, and rhamnose—Rha 0.94 %), as we expected since xylan and pectic epitopes were more abundant in the glycome profiling for these extracts.

In the carbonate extract, Xyl accounts for ~75 % of total monosaccharides, as expected because of the increased high amount of xylan and low xyloglucan epitopes observed in the glycome profiling. However, the total Glc is higher (~10 %) than expected by observations of glycome profiling, this probably reflects residual starch in this extract. Gal accounts for ~7 % of the yields, followed by Ara, ~3.5 %, Rha and mannose (Man), ~1.5 %, and Fuc, ~0.6 %.

Although, the 1 M and 4 M KOH solutions have similar release properties, the monosaccharide abundance found in these extracts was different, although Xyl being the most abundant monosaccharide in both (~94.5 % for 1 M and ~81.5 % for 4 M). In the 1 M extract, Gal was the second most abundant monosaccharide (~2.5 %), while the others account for ~3 %. In these extracts, Gal has the lowest contribution (in percentage) among the monosaccharides compared with the other extracts, which is in accordance with the lower abundance of pectic epitopes observed in the glycome profiling. Glc was the second most abundant monosaccharide (~9 %) in 4 M, as expected because of the high abundance of xyloglucan epitopes observed in the glycome profiling. The contributions of the other monosaccharide were as follows: Man ~4 % and Gal ~3 %, with the others accounting for ~2.1 %.

After lignin removal (chlorite and 4 M PC extracts), the polysaccharides that were strongly attached to the cell walls are now abundant in these extracts. The abundance of Xyl is higher for 4 M PC (~73.5 %) than it is for chlorite Xyl (~52 %), as we also observed xylan enrichment in the glycome profiling. Gal is the second most abundant monosaccharide in both extracts (~30 % for chlorite and ~10 % for 1 M KOH). The other monosaccharides found in the chlorite extract were as follows: Ara (~7.4 %), Rha (~2.8 %), Man (~1.4 %), and Fuc (~0.35 %). The Glc abundance in the chlorite extract was statistically different between the species (lower in *E. globulus* and higher in *E. urophylla*), probably related with the xyloglucan differential abundance also found in the glycome profiling. Significant differences were also observed in the 4 M PC extract for Ara, which was more abundant in *E. globulus*, and Xyl, which was more abundant in *E. urophylla*.

**Table 2** Monosaccharide yields in extracts

| | Fucose | Arabinose | Galactose | Rhamnose | Glucose | Xylose | Mannose | Fucose | Arabinose | Galactose | Rhamnose | Glucose | Xylose | Mannose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ammonium oxalate | | | | | | | 4 M KOH | | | | | | |
| *E. globulus* | 0.17 | 2.81 | 4.29 | 0.91 | 60.96 | 26.71 | 4.15 | 0.39 | 1.04 | 3.09 | 0.95 | 10.03 | 81.00 | 3.49 |
| *E. grandis* | 0.07 | 2.68 | 10.23 | 1.03 | 58.67 | 24.01 | 3.30 | 0.17 | 1.08 | 3.06 | 0.93 | 8.53 | 81.88 | 4.36 |
| *E. urophylla* | 3.14 | 5.05 | 9.26 | 0.88 | 59.44 | 19.96 | 2.28 | 0.18 | 1.11 | 3.88 | 0.72 | 8.79 | 81.72 | 3.60 |
| *p* value | *0.40* | *0.36* | *0.47* | *0.92* | *0.96* | *0.33* | *0.30* | *0.36* | *0.53* | *0.44* | *0.55* | *0.24* | *0.43* | *0.84* |
| | Sodium carbonate | | | | | | | Sodium chlorite | | | | | | |
| *E. globulus* | 0.25 | 3.69 | 6.91 | 1.65 | 3.87 | 82.33 | 1.31 | 0.35 | 10.82 | 32.20 | 3.75 | 3.30 | 48.35 | 1.23 |
| *E. grandis* | 0.13 | 2.71 | 4.95 | 1.35 | 13.06 | 76.15 | 1.66 | 0.38 | 6.09 | 27.27 | 2.15 | 6.46 | 56.22 | 1.43 |
| *E. urophylla* | 1.48 | 3.75 | 8.49 | 1.55 | 14.56 | 68.57 | 1.61 | 0.30 | 5.70 | 27.09 | 2.74 | 9.97 | 52.67 | 1.54 |
| *p* value | *0.84* | *0.92* | *0.27* | *0.09* | *0.06* | *0.09* | *0.84* | *0.60* | *0.12* | *0.21* | *0.46* | *0.048** | *0.35* | *0.72* |
| | 1 M KOH | | | | | | | 4 M KOH PC | | | | | | |
| *E. globulus* | 0.03 | 0.45 | 2.74 | 1.09 | 0.67 | 94.35 | 0.69 | 0.29 | 5.87 | 10.43 | 0.94 | 7.98 | 69.40 | 5.10 |
| *E. grandis* | 0.17 | 0.34 | 2.23 | 0.89 | 0.67 | 95.12 | 0.57 | 0.20 | 3.40 | 10.23 | 0.41 | 6.34 | 74.97 | 4.46 |
| *E. urophylla* | 0.03 | 0.36 | 2.58 | 0.89 | 1.66 | 93.89 | 0.52 | 0.39 | 2.44 | 8.43 | 0.66 | 7.32 | 76.51 | 4.25 |
| *p* value | *0.24* | *0.21* | *0.61* | *0.06* | *0.12* | *0.38* | *0.08* | *0.15* | *0.005** | *0.47* | *0.75* | *0.13* | *0.019** | *0.21* |

Mean of monosaccharide yields (in percentage) in each extract and *p* value between species. Significant differences are indicated by (*) ($p < 0.05$) by ANOVA test or Kruskal-Wallis test

### Lignin Analysis

The percentage of Klason lignin was estimated by NIR and significant differences were detected between *E. globulus* (~23.76) and the other two species: *E. grandis* (~24.97) and *E. urophylla* (~25.03) (Fig. 2a). This result was confirmed by wet chemical lignin analyses of *E. grandis* (22.04 Klason%; 25.64 total lignin) and *E. urophylla* (23.01 Klason%; 26.63 total lignin); *E. globulus* was not measured by this parameter due to lack of material available (data not shown). Py-lignin measured by analytical pyrolysis also had *E. globulus* with lower lignin content average (~22.51) compared to *E. grandis* (~24.23) and *E. urophylla* (~23.64), despite no statistical significance observed (Fig. 2b).

In Fig. 2b, significant differences at the S/G ratio can be observed. This represents the ratio between most monomers that form lignin between species, being higher in *E. globulus* (~1.71) compared to *E. grandis* (~1.50) and *E. urophylla* (~1.59).

We also analyzed lignin content and composition on adult trees for the same species. We found that S/G ratio was also higher on *E. globulus* and lower on *E. urophylla* (Lepikson et al., unpublished data).

### Enzymatic Hydrolysis and Non-structural Sugars

The accessibility of the cell wall sugars was investigated by enzymatic hydrolysis with *Neurospora Crassa* secretome in two scenarios: before and after DMSO treatment, which aims to remove the excess of starch from the samples (Fig. 3). We found that, before starch removal, the amount of reducing sugars produced in *E. grandis* is significantly higher than the other two species. However, after DMSO treatment, *E. globulus* had more reducing sugars produced than the other two species.

We also performed enzymatic hydrolysis with no pre-treatment and with alkali (NaOH) pre-treatment on adult trees from the same species, and *E. urophylla* is showed to be much more recalcitrant on both essays when compared to *E. globulus* and *E. grandis* (Lepikson et al., unpublished data).

Total starch content was measured in all samples before DMSO treatment (Fig. 4). We found significantly less starch accumulated in *E. globulus* young trees (~3 μg $g^{-1}$ DW) when compare with other species (~17.8 μg $g^{-1}$ DW).

Also, soluble sugars (glucose, fructose, raffinose, and sucrose) were also measured in samples before cell wall isolation (ethanol extraction) and we observed no significant differences among species (data not shown).

## Discussion

*Eucalyptus* species that have been planted to supply paper and pulp industries may now become important candidates for second-generation biofuel production, since they have

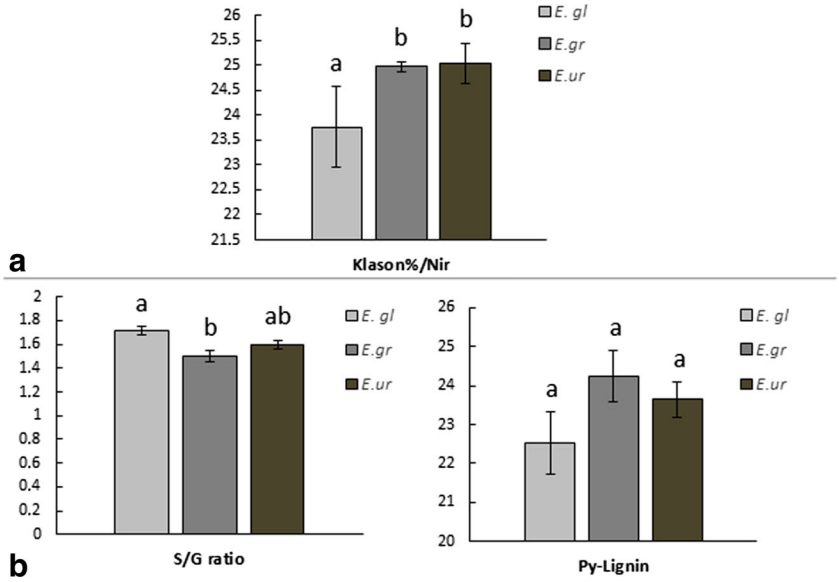


**Fig. 2** Lignin content and composition in *Eucalyptus* species. **a** Nir prediction of Klason (acid insoluble) lignin of young trees of *Eucalyptus* species. **b** Pyrolysis results for S/G ratio Py-lignin content (%) in the xylem of young trees of *Eucalyptus* species. Data are presented as mean ± SE of tree replicates. *Different letters* indicate significant differences between treatments ($p < 0.05$) by ANOVA test

undergone genetic improvement processes for years to achieve high pulp production. However, there are phenotypic differences in the wood of these species that drastically affect the production process. Thus, the knowledge of the fine structure of their cell walls is essential to guide the development of the process of lignocellulosic feedstock assessment.

The data produced here provides us insights into the model of connection between cell wall molecules of each of the species studied.

## Cell Wall Structure and Glycan Extractability in Eucalyptus Wood

Plant cell walls are a complex organized network composed mostly of cellulose, hemicelluloses, pectin, and lignin. These polymers are interconnected by several known connections [24, 29–33] and probably by a large number of interactions between molecules in the cell walls that still need to be discovered. In addition, wood cell walls vary greatly in composition and structure. Even in species in the same genus, as the ones studied here, variations occur and can be determinant in recalcitrance parameters [34].

HG, composed of galacturonic acid (GalA) residues, is the most abundant pectic polysaccharide in cell wall and in middle lamella [35, 36]. Within the cell wall, most GalA molecules are methyl-esterified [32], while HG, primarily present in the middle lamella, is de-methyl-esterified and the presence of divalent cations is responsible for forming interactions between adjacent carboxyl groups [37]. The HG backbone is covalently linked with rhamnogalacturonan I and II (RG-I and RG-II, respectively) [32]. When the ammonium oxalate salt is added to AIR samples, it forms precipitates with calcium and the bridges between de-methyl-esterified HGs are broken [32]. Thus, the pattern observed in *Eucalyptus* species for the ammonium extract is related to the HG possibly released from middle lamella and RG-I connected to HG (Fig. 1). We also

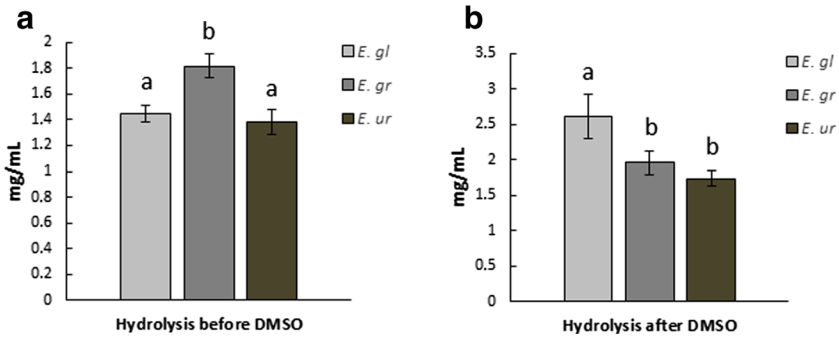


**Fig. 3** Enzymatic hydrolysis in *Eucalyptus* cell walls. Results of reducing sugar yields after enzymatic hydrolysis with *Neurospora crassa* secretome in two different scenarios: **a** Before DMSO treatment. **b** After DMSO treatment. *Different lower case letters* indicate significant differences between species ($p < 0.05$) by ANOVA test

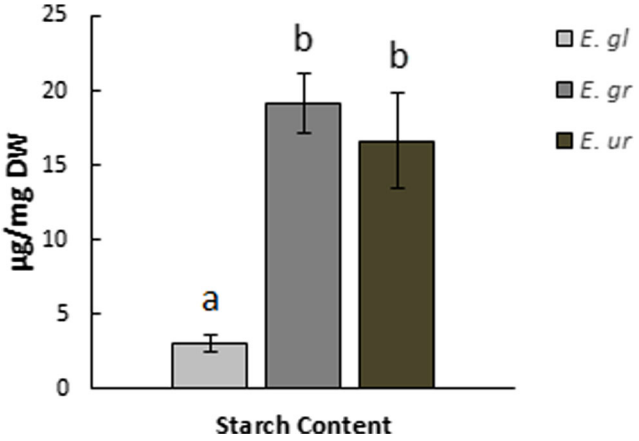


**Fig. 4** Starch content in *Eucalyptus* species. Starch content (µg $g^{-1}$ DW) in xylem of young trees of *Eucalyptus* species. Data are mean ± SE of tree replicates. *Different lower case letters* indicate significant differences between species ($p < 0.05$) by ANOVA test

observed high Glc abundance in the ammonium extract (Table 2). This probably happened because starch in the wood tissue might have been resistant to extraction with DMSO.

Sodium carbonate is an alkaline solution that has the property to extract pectins and also hemicelluloses that are loosely attached to the wall [38]. We observe in Fig. 1 and Table 1 that XG epitopes are more abundant in *E. urophylla* in relation to other species. In a previous work performed for adult trees [39], we have found that based on gene expression analysis, *E. urophylla* probably builds less hemicelluloses than the other two species. Although our results demonstrate that XG, in carbonate and also chlorite extracts, is more easily extracted from *E. urophylla*, its influence on production of fermentable sugars is lower, since XG is the most abundant type of hemicellulose in the primary cell walls of dicotyledon plants [40]. Thus, as already mentioned above, woody feedstock stores, in their secondary cell walls, a large amount of fermentable sugars [4, 5], which are mainly stocked in cellulose and xylan [40].

The removal of strong interactions between hemicelluloses and cellulose (that occur by hydrogen bonds) can be achieved by treatments with harsher solutions of 1 M and 4 M KOH [41]. Xylans are a large group of hemicelluloses, formed by β-1,4 xylose links and different type of substitutions, mostly present in wood secondary cell walls. A higher abundance of xylan epitopes is present both in 1 M and 4 M and 4 M PC, which highlights the importance of these molecules in cell wall architecture, as already mentioned above [30–33]. In addition, in the harsher extracts (4 M and 4 M PC), we observe xyloglucan epitopes, which means that part of this hemicellulose is strongly adhered to the walls. We observed in the monosaccharide composition the same pattern of abundance: some Fuc, Glc (~6–9 %), xylose (~50–80 %), and Gal (~3–30 %) in relation to other monosaccharides (Table 2) in 4 M, chlorite, and 4 M PC extracts that indicated the presence of XG in these extracts.

Lignin is mostly deposited in the secondary cell walls, but it can also be found in the middle lamella [32]. It interacts with the cell wall polysaccharides through several connection types called "lignin-carbohydrate complex" (LCC) [32]. There is evidence that the ability of lignin to form covalent bonds with hemicellulose is stronger than with cellulose. The linking of lignin with pectin can also occur [32]. The addition of sodium chlorite and glacial acetic acid to the samples aims to break down the lignin polymers and their monomers are washed away. Pectic and hemicellulose epitopes were abundant in the extracts that followed the treatment with sodium chlorite (respecting some differences in abundance between species). Thus, the lignin removal would have exposed both the pectic and hemicellulose epitopes that were strongly linked to this polymer which became exposed and easily extracted.

The difference in abundance of XG and xylan (groups 1 to 3) epitopes between *E. urophylla* and the other species (this difference was also observed when comparing to populous [23] chlorite extract) may indicate that, during the process of delignification, the material loss associated with the process is greater in this species. In an industrial scale, it means great loss in productivity. However, after the addition of a new solution of 4 M KOH, the release of hemicelluloses was observed to be lower in *E. urophylla,* which indicates that the remaining polysaccharides present in the wall are closely attached to cellulose and the amount present in *E. urophylla* is lower. That is, as already inferred by the gene expression [39], the cellulose content in *E. urophylla* is probably lower than the other species. Therefore, the amount of wall polysaccharides that strongly adhere to the cellulose also tends to be lower.

As observed in Fig. 1, RG/AG and AG epitopes are distributed in all extracts, in most cases with differential abundance between species. For this reason, it is difficult to establish a pattern for the occurrence of these molecules and relate them to the differential construction of walls between species.

## Lignin and Sugar Accessibility

Lignin greatly impacts the accessibility of fermentable sugars. In *Eucalyptus* wood, lignin concentration is related not only to the cell wall composition in different species but also acts in response to environmental stresses [25]. Lignin is a polymer consisting of different monomers, especially those derived from coniferyl alcohol (guaiacyl—G), sinapyl alcohol (syringyl—S), and p-coumaryl alcohol (hydroxyphenyl—H) [42]. In *Eucalyptus*, lignin monomers are formed mainly by S and G, and containing a small amount of H. These monomers differ in the number of methylation, as the more methylated, the more easily extracted [43]. Thus, S is the most methylated monomer and H, the least methylated. Also, other results from our group showed that an S/G increase in *Eucalyptus* plants can result in greater amounts of reducing sugars as observed by enzymatic hydrolysis [44, 45]. Our results demonstrate that

*E. globulus* has significant lower lignin content when compared to *E. grandis* and *E. urophylla* and also has higher S/G ratio. This indicates that, the lignin present in *E. globulus* is more easily extracted when compared to the other species, accounting for the best wood quality for this species.

These results corroborate with our previous work with adult tree growth in Brazil [39] that evidenced contrasting gene expression patterns (less lignin content in *E. globulus*), and other results obtained from E*ucalyptus* trees from Australia [34] showed that *E. globulus* has less lignin among a group of *Eucalyptus* species. To verify the correspondence of results between young and adult eucalyptus trees, we also evaluated adult trees from the same species for lignin composition and extractability. We found similar results although less differences between *E. globulus* and *E. grandis* (Lepikson et al., unpublished data), that we attribute to the better adaptability of *E. grandis* to Brazilian climate, while *E. globulus* suffers environmental stress when it leaves the greenhouse.

Additionally, the amount of reducing sugars produced in *E. grandis* was high in the first scenario of enzymatic hydrolysis (without starch removal). The intermediate cell wall accessibility, as already observed in glycome profiling, and intermediate cellulose content, as previously discussed [18, 19, 39] are probably contributing to this result, as also, the high starch accumulated in *E. grandis* xylem cell walls (Fig. 4).

After starch removal (second enzymatic hydrolysis scenario), the amount of reducing sugars present in *E. globulus* is higher when compared with *E. grandis* and *E. urophylla*. This result can mean a better cell wall accessibility as well as greater amount of cellulose produced in wood [18, 19, 39]. As we can observe in Table 2, the Glc content is high and beyond the expectations based on glycome profiling results. For carbonate extract, the pattern observed for Glc content in samples is the same for the starch content in Fig. 4, which is in agreement with the hypothesis that, in *Eucalyptys* wood, starch may be resistant to DMSO extraction. So, results concerning starch removal should be understood here as mostly starch removal.

It is important to note that in any of the scenarios presented, *E. urophylla* has some disadvantage over the other species, showing its undesirable characteristics regarding cell wall accessibility and sugar production.

## Conclusion

In general, the sugar and lignin contents in *Eucalyptus* species evaluated in this work were found to differ in many aspects. *Eucalyptus* cell wall molecules are linked in two different scenarios: (1) molecules that are loosely connected, making *Eucalyptus* xylem cells easily accessible to pre-treatment steps usually applied in biofuel production and (2) molecules tightly connected in particular ways in the cell walls of each species. The lignin content is lower in *E. globulus* and its higher S/G ratio makes it easily extracted in comparison with the other two species studied [34, 44, 45]. The amount of reducing sugars produced after hydrolysis on *E. globulus* cell walls (after removal of starch excess) was higher than the other two species, while *E. uroplylla* has the worse productivity. The linkages between lignin and cell wall carbohydrates seem to hamper cellulose release from the walls. Hemicelluloses are tightly connected with lignin in *E. urophylla* cell walls (and probably with cellulose too), resulting in great losses of fermentable sugars during the cellulose isolation process. This knowledge is crucial regarding the expansion of *Eucalyptus* products in order to reach second-generation ethanol with a high yield.

## Materials and Methods

We compared the carbohydrate profile of xylem secondary walls of three *Eucalyptus* species with different wood characteristics (young trees). We estimated lignin content and composition through the Klason method, NIR spectroscopy, and analytical pyrolysis. We also measured total starch accumulated in samples and cell wall accessibility by enzymatic hydrolysis. To establish and quantify the interrelationships among the wall polymers, we evaluated cell wall deconstruction though glycome profiling and we measured monosaccharides in fractionated cell walls. These data were linked to the gene expression profile of adult plants of the same species previously described by Salazar et al. [39] and other results also obtained for adult tress (Lepikson et al., unpublished data).

### Plant Material

Plants of three *Eucalyptus* species (*E. globulus*, *E. grandis*, and *E. urophylla*) were grown in a greenhouse (three plants of each species being approximately 1 year old) at Unicamp/Brazil and watered three times a week. Xylem samples were collected after bark removal and immediately frozen in liquid nitrogen, lyophilized, and then pulverized in a ball mill.

### Preparation and Fractionation of Alcohol-Insoluble Residue

For AIR preparations, 500 mg of each pulverized xylem sample were resuspended in 30 ml of 80 % ethanol and incubated at 80 °C for 20 min. The samples were then centrifuged at 3000 g for 15 min and the supernatant was discarded. This step was repeated four times and the samples were washed twice with distilled water to remove excess ethanol. The resulting material was resuspended in 30 ml of 90 % DMSO and incubated at room temperature for 12 h with constant stirring in order to remove the starch. Samples were then centrifuged, the supernatant was discarded and new extraction with 90 % DMSO was performed for 2 h. The samples were centrifuged again and the

supernatant was discarded. Washing with distilled water was performed six times for the complete removal of DMSO. The isolated cell walls were lyophilized for 24 h and weighed in order to measure the amount of waste removed [24, 43, 46].

For cell wall fractionation, 200 mg of AIR samples were submitted to sequential extractions of chemicals with increasing harshness as described by Pattathil et al. [28]. The fractions were named after the chemicals used in extraction as follows: ammonium oxalate (named ammonium), sodium carbonate (carbonate), 1 M KOH (1 M), 4 M KOH (4 M), sodium chlorite (chlorite), and 4 M KOH post chlorite (4 M PC). The sodium carbonate and KOH solutions were prepared with 0.5 % sodium borohydride [28].

All cell wall extracts were dialyzed against four changes of distilled water (sample: water ~ 1:60) at room temperature under mild constant stirring for a total of 60 h. Each change of distilled water followed a minimum of 6 h. The samples were then transferred to new 50 ml tubes and then lyophilized.

### Glycome Profiling

Total sugar estimation was performed in duplicates for each sample through phenol-sulfuric acid colorimetric assay adapted from Dubois et al. [47] and Masuko et al. [48]; samples were read (ELISA plate reader) at 490 nm wavelength.

Glycome profiling analyses [28] of the cell wall extracts were performed using ELISA test with a large library of cell wall glycan-directed monoclonal antibodies [28, 49]. The technique employs a toolkit of 155 monoclonal antibodies that recognize cell wall carbohydrates (except RG-II and cellulose) to access their strength of association to the cell wall [28, 49]. The carbohydrates recognized are the following: pectin and pectic-arabinogalactan—homogalacturonan (HG) backbone-1 and 2 (referred as HG group), rhamnogalacturonan (RG)-1 backbone, RG-1b, RG-1c (referred as RG group), RG-1/arabinogalactan (referred as RG/AG group) and AG-1, AG-2, AG-3 and AG-4 (referred as AG group); hemicelluloses—non-fucosylated xyloglucan (referred as Non-fuc XG group), fucosylated xyloglucan (referred as Fuc-XG group), xylan-1 to xylan-7 (referred as xylan group), and galactomannan (referred as galactom group). Epitopes for acetylated mannan, β-1,3 glucan, linseed mucilage RG-1, and physcomitrella pectin were weakly recognized by the antibodies and were not considered in this study.

### Monosaccharide Determination

Approximately 2 mg of cell wall extracts were hydrolyzed with 100 μL of 72 % $H_2SO_4$ (*v*/*v*) for 45 min at 30 °C. The acid was diluted to 4 % and the material was autoclaved for 1 h at 121 °C [50]. Samples were neutralized with NaOH and deionized through cation and anion exchange columns (Dowex). Subsequently, they were analyzed by HPAEC-PAD on a CarboPac SA10 column 4 × 50 mm (DX-500 system, Dionex®) using a mixture of 99.2 % water and 0.8 % (*v*/*v*) 150 mM NaOH as eluent (1 mL $min^{-1}$), in a 12-min room temperature. The monosaccharides Fuc, Ara, Gal, Rha, Glc, Xyl, and Man were detected with a post-column a CarboPac SA10 column 4 × 250 mm addition of 500 mM NaOH (1 mL $min^{-1}$) [24].

### Analytical Pyrolysis

Analytical pyrolysis of xylem AIR samples was performed according to the protocol previously described by Lepikson-Neto et al. [44].

### Lignin Determination

Klason (acid insoluble lignin) was determined from a pool of three biological samples and performed as described by Lepikson-Neto et al. [44], it was also predicted utilizing NIR-PLSR models as described at Camargo et al. [25].

### Enzymatic Hydrolysis

Hydrolysis was performed as described at Lepikson-Neto et al. [45].

### Non-structural Carbohydrates Determination

The extraction of soluble sugars were performed with 10 mg of dry pulverized material in 1.5 ml of ethanol (80 % *v*/*v*) for 20 min (80 °C) [51], this step was repeated four times. The extracts were dried and resuspended for quantitative and qualitative analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) using a Dionex-DX500 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CARBOPACPA1 column using 100 mM NaOH as eluent with a 1-mL $min^{-1}$ flow rate [52].

The starch extraction was performed by an enzymatic method [53] as described below: to the milled *Eucalyptus*, xylem samples were added 0.5 mL (120 U $ml^{-1}$) of α-amylase, diluted in 10 mM MOPS buffer pH 6.5. The samples were incubated at 75 °C for 30 min. This procedure was repeated to a total of 120 units of enzyme. The samples were cooled to 50 °C, and then 0.5 mL of a solution containing 30 U $ml^{-1}$ of amyloglucosidase in sodium acetate buffer 100 mM pH 4.5 was added. The samples were incubated at 50 ° C for 30 min. This procedure was repeated. After these incubations, 100 mL of 0.8 M perchloric acid (to stop the enzymatic reaction and precipitate proteins) were added. After a quick centrifugation (2 min 9300*g*), the starch content determination was performed by quantifying the glucose in the starch hydrolysis process. To these aliquots, 300 mL of reagent Glucose PAP Liquiform (CENTERLAB) was added.

After incubation for 15 min at 37 °C, the glucose content was determined by spectrophotometer coupled to ELISA reader at wavelength of 490 nm. To prepare the standard curve, a glucose solution (SIGMA) with concentrations of 0, 1, 2.5, 5.0, 7.5, 10, and 12.5 mg/mL was used.

### Statistical Analysis

For all experiments, three unrelated trees of each species were analyzed. All data were tested for normality by the Anderson-Darling test ($p < 0.05$); for homogeneity of variance, we used Levene's and Bartlett's test ($p < 0.05$) and the symmetry was given by the value of skewness allowing a variation between +2 to −2. If the variances were homogeneous, we used the two-way ANOVA test; otherwise, the nonparametric Kruskal-Wallis test was used. Differences were considered significant at $p$ value <0.05.

**Acknowledgments** The authors would like to thank International Paper-Brazil for kindly providing plant materials and Espaço da Escrita/Coordenadoria Geral-UNICAMP for English revision. This work was supported by research funding of FAPESP (process number 2007/54877-0) and grants from International Paper do Brasil (IP/IB/Gene Discovery: 3972).

## References

1. Buckeridge MS, Goldman GH (2011) Routes to cellulosic ethanol. Springer, New York
2. Hochman G, Zilberman D (2014) Algae farming and its bio-products. In: Edited by McCann MC, Carpita NC, Buckeridge MS, editors. New York: Springer, 40–66
3. Meyer MM and Salatino A (2014) Perspectives in Brazil of the contribution of palm trees to biodiesel production. In: McCann, MC, Carpita, NC, Buckeridge, MS, editors. New York: Springer, 141–152
4. Ragauskas AJ et al (2006) The path forward for biofuels and biomaterials. Science 311:484–489
5. Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels, Bioproducts and Biorefining 1(2):119–134
6. Salisbury FB, Ross CW (1991) Plant physiology, 4th edn. Wassworth Publishing Company, Belmont
7. Li L, Lu S, Chiang V (2006) A genomic and molecular view of wood formation. Crit Rev Plant Sci 25:215–233
8. Carpita NC, Campbell M, Tierney M (2001) Plant cell walls. Springer Science + Business Media, Dordrecht
9. Wilson SM et al (2012) Pattern of deposition of cell wall polysaccharides and transcript abundance of related cell wall synthesis genes during differentiation in barley endosperm. Plant Physiol 159:655–670
10. Hamelinck CN, Hooijdonk G, Faaij APC (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28:384–410
11. Simmons BA, Loque D, Blanch HW (2008) Next-generation biomass feedstocks for biofuel production. Genome Biol 9:242
12. Naik SN, Goud VV, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sustain Energy Rev 14:578–597
13. Li M, Pattathil S, Hahn MG, Hodge DB (2014) Identification of features associated with plant cell wall recalcitrance to pretreatment by alkaline hydrogen peroxide in diverse bioenergy feedstocks using glycome profiling. RSC Advances 4:17282
14. Turnbull JW (2001) Economic and social importance of eucalyptus. In: Keane PJ, Kile GA, Podger FD, Brown BN, editors. Collingwood: CSIRO Publishing, 1–9
15. González-García S, Moreira MT, Feijoo G (2012) Environmental aspects of eucalyptus based ethanol production and use. Sci Total Environ 438:1–8
16. Potts BM and Pederick LA (2000) Morphology, phylogeny, origin, distribution and genetic diversity of eucalyptus. In Keane PJ, Kile GA, Podger FD, Brown BN, editors. Collingwood:CSIRO Publishing. 11–34
17. Foekel C (2009) Papermaking properties of eucalyptus trees, woods, and pulp fibers. [http://www.eucalyptus.com.br/eucaliptos/ENG14.pdf,] Accessed in 13 Sep 2014
18. Grattapaglia D (2008) Genomics of *Eucalyptus*, a global tree for energy, paper, and wood. In Moore PH, Ming R, editors. New York: Springer, 259 – 297
19. Grattapaglia D (2004) Integrating genomics into Eucalyptus breeding. Genet Mol Res 369–379
20. Bison O, Ramalho MAP, Rezende GDSP, Aguiar AM, De Resende MDV (2007) Combining ability of elite clones of Eucalyptus grandis and Eucalyptus urophylla with Eucalyptus globulus. Genet Mol Biol 30(2):417–422
21. Silveira RL, Stayanov SR, Gusarov S, Skaf MS, Kovalenko A (2013) Plant biomass recalcitrance: effect of hemicellulose composition on nanoscale forces that control cell wall strength. J Am Chem Soc 135:19048–19051
22. Zeng J, Singh D, Gao D, Chen S (2014) Effects of lignin modification on wheat staw cell wall deconstruction by *Phanerochaete chrysosporium*. Biotechnology for Biofuels 7:161
23. DeMartini JD et al (2011) Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production. Energy Environ Sci 4(4332)
24. De Souza AP, Leite DCC, Pattathil S, Hahn MG, Buckeridge MS (2013) Composition and structure of sugarcane cell wall polysaccharides: implications for second-generation bioethanol production. Bioenergy Research 6:564–579
25. Camargo ELO et al (2014) Contrasting nitrogen fertilization treatments impact xylem gene expression and secondary cell wall lignification in *Eucalyptus*. BMC Plant Biol 14:256
26. De Souza AP et al (2015) J Exp Bot. doi:10.1093/jxb/erv183
27. Pattathil S, Saffold T, Gallego-Giraldo L, O'Neill M, York WS, Dixon RA, Hahn MG et al (2012) Changes in cell wall carbohydrate extractability are correlated with reduced recalcitrance of HCT downregulated alfalfa biomass. Ind Biotechnol 8(4)
28. Pattathil S, Avci U, Miller JS, Hahn MG (2012) Immunological approaches to plant cell wall and biomass characterization: glycome profiling. In: Himmel M (ed). New York: Humana Press, 61-72
29. McCann MC, Wells B, Roberts K (1990) Direct visualization of cross-links in the primary plant cell wall. J Cell Sci 96:323–334
30. Brett C, Waldron KN (1996) Physiology and biochemistry of plant cell walls, 1st edn. Champ and Hall, London
31. Popper ZA, Fry SC (2008) Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall. Planta 227(4):781–794
32. Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A (2011) Plant cell walls—from chemistry to biology. Garland Science, New York
33. Park YB, Cosgrove DJ (2015) Xyloglucan and its interactions with other components of the growing wall. Plant Cell Physiology 56(2): 180–194
34. Cetinkol OP et al (2012) Structural and chemical characterization of hardwood from tree species with applications as bioenergy feedstocks. PLoS One 7(12):e52820
35. Mohnen D (2008) Pectin structure and biosynthesis. Curr Opin Plant Biol 11:266–277

36. Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosec production and processability. Plant Sci 178(2):61–72
37. Liners F, Letesson J, Didembourg C, Cutsem PV (1989) Monoclonal antibodies against pectin recognition of a conformation induced by calcium. Plant Physiol 91:1419–1424
38. Carpita NC (1984) Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali. Phytochemistry 23(5):1089–1093
39. Salazar MM et al (2013) Xylem transcription profiles indicate potential metabolic responses for economically relevant characteristics of Eucalyptus species. BMC Genomics 14:201
40. Sheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289
41. Melton LD, Smith BG (2005) Isolation of plant cell walls and fractionation of cell wall polysaccharides. In: Wrolstad RE, Acree TE, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF, Smith D, Sporns P. New York: Wiley-Interscience, 697–719
42. Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546
43. Shi R, Sun Y-H, Li Q, Heber S, Sederoff R, Chiang VL (2010) Towards a systems approach for lignin biosynthesis in *Populus trichocarpa:* transcript abundance and specificity of the monolignol biosynthetic genes. Plant Cell Physiol 51(10):144–163
44. Lepikson-Neto J et al (2013) Flavonoid supplementation reduces the extractive content and increases the syringyl/guaiacyl ration in *Eucalyptus grandis* X *Eucalyptus urophylla* hybrid species. Bioresources 8(2):1747–1757
45. Lepikson-Neto J et al (2014) Flavonoid supplementation affects the expression of genes involved in cell wall formation and lignification metabolism and increases sugar content and saccharification in the fast-growing *eucalyptus* hybrid *E. urophylla x E. grandis*. BMC Plant Biol 14:301
46. Carpita NC (1983) Hemicellulosic polymers of cell-walls of *Zea* coleoptiles. Plant Physiol 72:512–521
47. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Calorimetric method determination of sugars and related substances. Anal Chem 28(3):350–356
48. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339(1):69–72
49. Pattathil S et al (2010) A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. Plant Physiol 153:514–525
50. Saeman JF, Harris EE, Kline AA (1945) Quantitative saccharification of wood and cellulose. Anal Chem 17:35–37
51. Buckeridge MS, Dietrich SMC (1996) Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of Sesbania marginata Benth (Leguminosae-Faboideae). Plant Sci 117:33–43
52. Dos Santos HP, Purgatto E, Mercier H, Buckeridge MS (2004) The control of storage xyloglucan mobilization in cotyledons of *Hymenaea courbaril*. Plant Physiol 135:287–299
53. Amaral LIV, Gaspar M, Costa PMF, Aidar MPM, Buckeridge MS (2007) A new rapid and sensitive enzymatic method for extraction and quantification of starch in plant material. Hoehnea. 34