Comparison of macromolecular interactions in the cell walls of hardwood, softwood and maize by fluorescence and FTIR spectroscopy, differential polarization laser scanning microscopy and X ray diffraction

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Abstract

We compared interactions between macromolecules in the cell walls of different plant origin, namely spruce wood (*Picea omorika* (Pančić) *Purkiňe*) as an example of softwood, maple wood (*Acer platanoides* L.) - a hardwood and maize stems (*Zea mays* L.) - a herbaceous plant from the grass family and widely used agricultural plant. We compared macromolecules' interactions in isolated cell walls from the three species by using Fourier transform infrared spectroscopy, X-ray diffraction and fluorescence spectroscopy. We also observed linear dichroism of the cell walls by using Differential Polarization Laser Scanning Microscope (DP-LSM), which provides information of macromolecular order. This method has not been previously used for comparison of the cell walls of various plant origins. It was shown that the maize cell walls have higher amount of hydrogen bonds that lead to more regular packing of cellulose molecules, simpler structure of lignin, and to a higher crystallinity of the cell wall in relation to the walls of woody plants. DP-LSM and fluorescence spectroscopy results indicate that maize has simpler and more ordered structure than both woody species. The results of this work provide new data for comparison of the cell wall properties that may be important for selection of appropriate plant for possible applications as a source of biomass. This may be a contribution to development of efficient deconstruction and separation technologies that enable release of sugar and aromatic compounds from the cell wall macromolecular structure.

Introduction

Plant cell walls (CW) represent the most abundant, renewable and biodegradable composite on Earth. It forms a large part of the plant body and define its characteristics. The specific form and function of the cell walls and interaction with the environment are based on variation in its chemical composition and connections between the building macromolecules. The secondary cell walls are rich in biopolymers such as cellulose, hemicellulose and lignin, which have opened the door to the development of wide applications in different industries, especially for biofuels and biomaterials (Pauly and Keegstra 2008; Ragauskas et al. 2006).

The major biopolymers in CW of plants are held together by a combination of covalent and non-covalent bonds to form a highly complex structure. The most important features are interactions between polysaccharides, those between lignin and polysaccharides, and the degree of crystallinity of cellulose and size of cellulose microfibrils.

The most abundant component in plant cell walls is cellulose. Numerous hydroxyl groups in cellulose are involved in complex intra- and inter- molecular hydrogen bonds which give high-order (crystalline) and low-order (amorphous) regions (Kondo 2004; Wang 2008). Amorphous regions of cellulose microfibrils interact with hemicellulose chains (Atalla et al. 1993; Cosgrove 2005). Hydrophilic character of the cellulose surface is dependent on hydroxyl group distribution on the surface (geometry) together with their ability to maximize hydrogen bonds (Perez and Mazeau 2005). Hemicelluloses contribute to strengthening of the cell wall by interaction with cellulose and lignin (Atalla et al. 1993; Barakat et al. 2007; Ruel and Joseleau 2005; Scheller and Ulvskov 2010). They influence organization of cellulose via hydrogen bonds as a major non-covalent interaction in the cell walls (Atalla et al. 1993; Perez and Mazeau 2005) providing a contact between cellulose microfibrils and cell wall matrix polymers. The main difference among hemicelluloses of hardwood and softwood is in the content of xylan and glucomannan, a greather content of xylan being in hardwood and greather content of glucomannan in softwood. Arabinoxylans are the main hemicellulosic polysaccharides in graminaceous species and in grasses (Christensen et al. 2010). Lignins enable vascular plants to form their water/nutrient conducting cells and to provide a mechanism for withstanding compressive forces. Lignin composition varies in different groups of plants being guaiacyl- (G), guaiacyl/syringyl (GS), and hydroxyphenyl/guaiacyl/syringyl-type (HGS) lignin characteristic for softwoods, hardwoods, and graminaceous plants, respectively (Sun et al. 2012). Based on previous research, cellulose fibers and polysaccharide matrix can influence monolignol polymerization and ultrastructural organization of lignin in the plant cell walls (Houtman and Atalla 1995; Micic et al. 2000).

The chemical compositions vary between hardwood and softwood as well as among individual species. In general, softwood species have a comparable cellulose content (40–44%), higher lignin (26–34%), and lower hemicellulose content (20–32%) as compared to hardwood species (cellulose 40–44%, lignin 23–30%,

hemicellulose 15–35%; Haygreen and Bowyer 1996; Rowell et al. 2000). In graminaceous species, such as maize, contents of cellulose, hemicellulose and lignin are 42-43 %, 30% and 21-22%, respectively (Lv et al. 2010).

Our research compares secondary cell walls of three different species, spruce wood (*Picea omorika* (Pančić) *Purkiňe*) as an example of softwood, maple wood (*Acer platanoides* L.) as an example of hardwood and maize stems (*Zea mays* L.) as an example of herbaceous plant from the grass family and also a widely used agricultural plant. We compared chemical composition of isolated cell walls and polymer interactions by using several parameters: monomer interactions by FTIR (Fourier transform infrared) spectroscopy, cellulose crystallinity by XRD (X-ray diffraction) method, and lignin autofluorescence by fluorescence spectroscopy combined with spectral deconvolution. We also observed fluorescence detected linear dichroism (FDLD) of cellulose labeled with Congo Red in the cell walls, by using Differential Polarization Laser Scanning Microscope (DP-LSM), which provides information of structural order. These two fluorescence methods have not been previously used for comparison of the cell walls of various plant origins. Our goal was to provide new information about structural characteristics of the isolated cell walls among these species which may be a basis for their possible applications, such as biofuel and biomaterial industry. This may be a contribution to development of efficient deconstruction and separation technologies that enable release of sugar and aromatic compounds from the cell wall macromolecular structure. Maize, softwood and hardwood species are abundant in the region of Western Balkan. Also, we demonstrate use of some new combinations of methods, such as fluorescence spectroscopy with spectral deconvolution and FDLD, for fast screening of differences among the cell walls of various plant origins.

Materials and methods

All reagents and substances used in the experiments were analytical grade and obtained from Sigma (Germany) and Fluka (Germany).

Plant material

Three different plant species have been used. The cell walls were isolated from branches of spruce (*Picea omorika* (Pančic) *Purkyne*, Gymnospermae, conifer), from branches of maple (*Acer platanoides* L., Angiospermae, Dicotyledones, deciduous species) and from maize stems (*Zea mays* L., Angiospermae, Monocotyledones, grains from the grass family). We chose branches since they are waste material from woods, and maize stems are also waste material.

The branches were taken at a height of 2 m above ground level from trees of similar ages (50–60 years old). The middle part of a straight branch at a point 1 m away from the trunk was used. Samples were taken from the outermost annual rings of a branch. At this position, it may be considered that the branch is composed of mature wood. The shape of the cross section of the branch that was used was round, with rings that were almost concentric, indicating that there were insignificant levels of reaction wood in the samples used.

Isolation of plant cell wall

The extractive-free cell wall material was obtained from the spruce branches, maple branches and maize stem. After removing the bark, the wood from the outermost annual rings were sliced into sticks. The maize stems were chopped into small pieces. Plant material (1 g) was homogenized in 10 mL of 80% methanol in 50 mL Big Clean tubes filled with a stainless steel matrix for 45s at a speed of 4.5 m/s, using a FastPrep-24 apparatus (MP Biomedicals, Santa Ana, CA, USA). After stirring for 5 min at room temperature, the sample was again subjected to FastPrep homogenization at the same speed. Thus obtained plant material was dried for 72 h at 80 °C. Dry homogenatate of plant material was ground into a fine powder. To obtain cell walls, 400 mg of powder was homogenized for 5–10 min in 10 ml 80 % methanol. The homogenate was slightly stirred for 1 h at room temperature and centrifuged for 5 min at 1500 × g. Further, the resulting precipitate was extracted twice with 10 ml 80 % methanol. The precipitate was subjected to the following washing steps, according to Strack et al. (1988) and Chen et al. (2000): 1× (1 M NaCl, 0.5 % Triton X-100), 2× distilled water, 2 × 100 % methanol, 2 × 100 % acetone (each step in 20 ml, 30 min). In each washing step, the sample was homogenized in 20 mL of corresponding solvent and then subsequently stirred for 10 min at room temperature and then centrifuged at 1500 x g for 10 min. The supernatant was subsequently removed. We used a FastPrep-24 System (MP Biomedicals, Santa Ana, CA, USA) in each isolation step for more efficient extraction of the cell wall material. The FastPrep-24 has long been used for the lysis and homogenization of plant tissues, prior to molecular applications (Haymes et al. 2004) such as nucleic acid isolation. The FastPrep-24 instruments and matrix tubes provide rapid and thorough, automated disruption of plant cell walls, which are difficult to homogenize/lyse.

FTIR spectroscopy and X-ray diffraction

The FTIR spectra of the dry cell wall samples were recorded by attenuated total reflectance (ATR), using a Nicolet 6700 spectrometer (Thermo Scientific, USA). The internal reflection element was a diamond ATR plate with angle of incidence 45 degrees. The sample area was 1.5 mm. Spectra were obtained at a resolution of 4 cm^{-1} and collected in transmission mode from 4000 to 400 cm^{-1}. Each background spectrum was obtained by averaging 16 recordings, and each sample spectrum was obtained by averaging 32 recordings. Such averaged spectra were used for evaluation or further processing (deconvolution).

For a better understanding of the structure of the OH-vibrations in samples, deconvolution of the FTIR spectral region from 3000 cm^{-1}- 3800 cm^{-1} was carried out with Gaussian contours (Ciolacu 2007, Ciolacu et al. 2011; Popescu et al. 2007, 2009, Yuan et al 2013). Nonlinear fitting of all FTIR spectra was performed using the Nelder–Mead simplex algorithm implemented in Matlab 6.5. The reduced chi-squared value for all the deconvoluted curves was $\chi^2<0.1$; therefore, the use of this function is a good approach. We chose to apply four components analysis after performing a series of deconvolutions where we varied the number of components from 1 to 5 and plotted the reduced χ^2 value as a function of the number of components. We found that reduced χ^2 decreased steadily in the log-log plot up to 4 components, after which stabilization was achieved (not shown). Therefore, no further progress in the quality of fitting was obtained by introducing the fifth component.

X ray measurements of dry isolated cell walls were carried out by Siemens D-500 powder diffractometer. CuK_α radiation was used in conjuction with a CuK_β nickel flter. We did repeated measurements for each type of sample (species), and we show representative difractograms. The percentage crystallinity of the samples was calculated from the ratio of the area under the diffraction peaks to the total area under the whole diffraction pattern (Georget et al. 1999; Hermans and Weidinger 1948). Amorphous background patterns were generated, fitted to, and subtracted from each diffraction pattern by using OriginPro 7.5.

Steady-state fluorescence spectroscopy

Fluorescence spectra were measured using a Fluorolog-3 spectrofluorimeter (Jobin-Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. Cell wall samples were measured in dry state, in a front-face configuration of the measuring cell. The slits for the excitation and emission beams were fixed at 4 and 2 nm, respectively. For spectral deconvolution, emission spectra of each sample were obtained by excitation at different wavelengths, starting from the excitation maximum at 360 nm, with 5 nm steps. Thus 20 spectra were collected for each species. Every single spectrum was obtained by averaging seven scans. The spectra were corrected for the dark current and the spectral sensitivity of the system. All measurements were performed at 25 °C and controlled by a Peltier element.

Deconvolution of spectra

In order to determine the number of components in an integral emission spectrum, which correspond to the number of different fluorophores in the structure of the cell wall, we applied a specific measuring procedure. Emission spectra of each sample were obtained by excitation at different wavelengths, starting from the excitation maximum

at 360 nm, with 5 nm steps. These were deconvoluted into a varying number of log–normal components, from three to five, using the log–normal method in order to find the optimal number of components for emission spectral analysis. Nonlinear fitting of all fluorescence spectra was performed using the Nelder–Mead simplex algorithm implemented in Matlab 6.5. For each sample, positions of component maxima, obtained for a series of spectra measured with a 5 nm step, were treated as random variables and corresponding histograms of component maxima positions were constructed. However, since histogram profiles (positions and relative amplitudes of histogram maxima) depended on the number of histogram abscissa intervals, we calculated the corresponding approximate probability density distribution (APD) by weighed averaging of histogram values for a set of histograms with varying abscissa intervals. For each sample we found an optimal log-normal number of components (three for maize, four for maple and five for spruce). Attempts with more components did not reduce error significantly (Kalauzi et al. 2007; Radotić et al. 2006).

DP-LSM

The confocal fluorescence intensity images were recorded on a Zeiss LSM 410 laser scanning microscope (Carl Zeiss Jena, Jena, Germany) equipped with a differential-polarization (DP) attachment (Garab et al. 2005; Steinbach et al. 2009). Briefly, the DP attachment modulates the polarization state of the excitation laser beam at 100 kHz between horizontally and vertically linear polarization, using a photoelastic modulator (PEM-90, Hinds Instruments). The PEM was placed between the beam expander and the main beam splitter. The FDLD signal, proportional to the fluorescence intensity difference, elicited by two orthogonally plane-polarized beams, was obtained from the demodulation circuit, and images were recorded on the LSM. Using the runtime calculation the FDLD imaging needs only a single scan and it avoids all the artefacts from the multiple scans (such as sample and light intensity stability, bleaching). The provided pixel values are the average of more than 10 cycles of modulation.

The images were in resolution of 512x512 dots, covering the area of 50x50 and 64x64 μm. Each image consists of two channels, FDLD channel and fluorescence emission channel. FDLD values for dipoles oriented along the Y-axis correspond to 1, while the values of dipoles oriented along the X axis correspond to -1. The FDLD values are in range from -1 to +1 due to the definition: $FDLD = (I_1-I_2)/(I_1+I_2)$. ($I_1$ fluorescence intensity was measured with vertically, I_2 with horizontally polarized excitation). The acquired 8 bit TIFF images were set to have a color scale from blue (#0000FF) to yellow (#FFFF00) for the FDLD channel.

The samples of isolated cell walls were stained with freshly prepared 2 % (w/v) solution of Congo Red (Merck, Darmstadt, Germany) for 30 minutes, followed by rinsing in distilled water three times. Congo Red has earlier been used to determine the mean cellulose fibril orientation in plants (Verbelen and Kerstens 2000). Samples (isolated cell wall fibrilar fragments) stained with Congo Red were excited at 488 nm and fluorescence emission was observed above 560 nm.

Results

FTIR spectroscopy

Figure 1 shows overlaid FTIR spectra for the three cell wall samples, in the region 1800 cm^{-1}- 700 cm^{-1} (Fig. 1a) and in the region 3600 cm^{-1}- 2800 cm^{-1} (Fig. 1b). Band at about 898 cm^{-1} in all three samples is characteristic for anomeric β-linkage of glucose molecules (Kacurakova et al. 2000). The region from 1100 cm^{-1}- 900 cm^{-1}, specific for the polysaccharides, gives a good overlap for all three cell wall samples. However, there is a slight shift in maximum band position, being 1016 cm^{-1} and 1022 cm^{-1} (C-O, C-C, C-C-O vibrations) for softwood and hardwood, respectively, and at 1024 cm^{-1} for maize sample. This is due to the fact that this region is sensitive to the axial and equatorial position of the (OH) groups in each sample which can affect quite significantly the band positions (Kacurakova et al.2000). In the maple there is a strong band at 1225 cm^{-1}- 1250 cm^{-1} while in the spectra of the other two species there is broad band at 1235 cm^{-1}- 1265 cm^{-1}. These bands are specific for lignin (Faix et al. 1991; Faix 1992; Liang and Marchesault 1959; Marchesault 1962). The bands at 1370 cm^{-1} and at 1420 cm^{-1}- 1430 cm^{-1} are characteristic for cellulose and may be used to determine its crystallinity, while the band at 898 cm^{-1} originates from the amorphous region in cellulose (Åkerholm et al. 2004; Ciolacu et al. 2011; Nelson and O'Connor 1964). Lignin-associated bands at 1500 cm^{-1}- 1515 cm^{-1} belongs to aromatic ring stretching vibration (Faix et al. 1991; Faix 1992) increase significantly in the samples of wood species, which is in accordance with the higher amount of lignin (Agarwal and Ralph 1997; Akerholm and Salmen 2001; Faix et al. 1991). The region between 1590 cm^{-1}- 1660 cm^{-1} is broad in all three samples and is specific for conjugated carbonyl/carboxyl group and for C=C bond in the benzene ring and in the side chains (Faix et al. 1991; Faix 1992; Pretsch et al. 1981). This band is very weak in the maize sample. In the carbonyl region, bands are found at 1720 cm^{-1}- 1730 cm^{-1}, corresponding to unconjugated C=O of esters and/or carboxylic acids. A strong broad band can be observed in the region of 3600 cm^{-1}- 3000 cm^{-1} (Fig. 1b), which is assigned to different OH stretching modes, and another band in the region of 3000 cm^{-1}- 2800 cm^{-1} is ascribed to the CH groups in cellulose and lignin (Adel et al. 2011; Faix et al. 1991). In spruce there are two well defined bands at 2848 cm^{-1} and 2912 cm^{-1} bands, specific for C-H stretching vibration in cell walls. In the maple and maize samples the CH band is broad and unstructured, indicating presence of many overlapping CH vibration modes.

In the broad region 3600 cm^{-1}- 3000 cm^{-1}, absorbance originates from OH vibrations in lignin (alcoholic and phenolic OH) and cellulose (Faix 1992; Fengel 1993; Ghaffar and Fan 2014; Liang and Marchessault 1959; Sarkanen and Ludwig 1971). A mixture of intermolecular and intramolecular hydrogen bonds is considered to cause broadening of the OH band in the FTIR spectra (Popescu et al. 2011) of all three samples. On the basis of previous research several characteristic spectral regions can be observed for hydrogen bonds in cellulose I, including two regions assigned to intra-molecular bonds, namely, O(2)H---O(6) (3460 cm^{-1}- 3405 cm^{-1}) and O(3)H---O(5) (3375 cm^{-1}- 3340 cm^{-1}), and one region assigned to intermolecular bonds, O(6)H---O(3) (3310 cm^{-1}- 3230 cm^{-1}). The region 3550 cm^{-1}- 3580 cm^{-1} is, characteristic for the free OH (6) and OH (2) (Fan et al. 2012; Popescu et al. 2007, 2011; Yuan et al. 2013). In this study the area-normalized intensity FTIR spectra of cell wall samples were analyzed. In order to differentiate the hydrogen bond type, Gaussian deconvolution model was applied in this spectral region (Fig. 2, Table 1). We performed a series of deconvolutions where we varied the number of components from 1 to 5. The four-component analysis provided the optimal result, giving the lowest reduced chi square value (<0.005). No further progress in the qualty of fitting was obtained by introducing the fifth component. According to the Figure 2, there are differences among the plant cell wall samples. Table 1 quantifies the result of the FTIR spectra deconvolution for the amounts of hydrogen bond OH stretching vibrations. It shows the total content of inter- and intra- molecular hydrogen bonds in the three cell wall samples. The highest content of intra-molecular hydrogen bonds (summary of O(2)H···O(6) and O(3)H···O(5)) was found in maize (70 %), while in maple and spruce this content is similar, 60 % and 58 % respectively. The inter-molecular hydrogen bonds in cell walls were most abundant in spruce (41 %), while in maple and maize was 36 % and 29 % respectively. Free OH (6) and OH (2) are present in very low amount in all samples.

X ray diffraction

By using X ray diffraction, crystallinity of cellulose was followed in the samples. Based on the parameters obtained from the diffraction patterns of the cell wall samples for maple, spruce and maize it is obvious that there is a difference in the overall crystallinity of the samples. Table 2 shows that crystallinity is nearly the same (42 %) in the cell walls of spruce and maple. Crystallinity of the cell wall of maize is much higher (58 %). The peaks obtained from the diffractograms are typical for cellulose I, and are located at $2\theta \approx 14.9, 16.49, 22.84$ (Marchessault and Sundararajan 1983). The peak at $2\theta \approx 23^{o}$ generally is described as "highly crystalline" region of cellulose, while a broad peak at $2\theta \approx 16^{o}$ is characteristic of the less organized polysaccharide structure (Fig. 3).

DP-LSM

Plant cell walls are highly organized fiber-laminate extracellular structures with strong anisotropy in their shape and growth and thus polarization microscopic tools have been widely used in revealing the anisotropic features of this complex cellulose-based structure (Baskin et al. 1999; Baskin 2005; Cosgrove 2005; Kerstens and Verbelen 2003). The FDLD observed by DP-LSM imaging is a suitable tool for mapping of the optical anisotropy of cellulose (marked by Congo red) in the cell wall that corresponds to the cell wall linear dichroism (Steinbach et al. 2008). We analysed distribution of linear dichroism (Steinbach et al. 2011) on the images of cell wall (Fig. 4). Width of obtained distribution curves indicates degree of linear order in the analysed structures. The distributions shows a quite similar orientation for the three species, however the narrowest is for maize, corresponding to simpler structure and more regular packing (observed parallel linear structures) of cellulose molecules than in the wood species.

Steady-state fluorescence spectroscopy

Figure 5 shows an example of the mathematical deconvolution of fluorescence spectra of cell walls isolated from maple, spruce and maize, excited at 370 nm. The overlaid normalized fluorescence emission spectra of softwood and hardwood cell walls, excited at 370 nm (Fig. 5a), do not differ in shape, but differ in spectral width. This indicates differences in the number of spectral components, implying variation in structural complexity among the two cell walls. The fluorescence spectra for maize sample are narrower than the spectra for woody species, indicating lower number of spectral components and thus implying simpler structure. For each sample we applied three, four and five component analysis, in order to get an optimal number of components and final position of the maxima and their grouping at appropriate wavelengths. Five-component model was optimal for analysis of fluorescent spectra for the cell wall of maple (Fig. 5b). Four-component analysis has proven to be the most appropriate for spectra of cell wall of spruce (Fig. 5c), and three-component decomposition for the sample of maize stem (Fig. 5d). As result of spectral deconvolution, an approximation of the probability density (APD) for component positions on the wavelength-axis was obtained, where an APD maximum corresponds to the position of a cell wall spectral component (Fig. 6), as shown in the previous studies of mathematical analysis of fluorescence spectra (Djikanović et al. 2012a,b; Donaldson et al. 2010; Kalauzi et al. 2007; Radotić et al. 2006). The spectral components are designated I – V (Fig. 6). Based on this analysis three spectral components were obtained for maize, corresponding to the three main emitting structures. In the maple and spruce, four and five components were obtained, corresponding to the four and five emitting species, respectively.

The component I (≈ 400 nm) appears in the high energy part of the spectrum only in softwood and hardwood samples. In the maize sample this component is absent. Component II (≈ 420 nm) is partly overlayed in all three samples. Component III (≈ 445 nm) is the main structural component from lignin macromolecule. It is mainly at the same position for cell wall of maize and maple, but slightly red-shifted in the sample of softwood cell wall. For component IV (≈ 480 nm) the APD diagram shows blue shift in the sample of cell wall of hardwood species. Component V (≈ 520 nm) is present only in spruce sample.

Discussion

Based on XRD technique we have shown the difference in crystallinity of the cell walls of the three plant species. It can be observed that the crystallinity is nearly the same (42 %) in the cell walls of spruce and maple (Table 2). Woody species show a crystallinity of about 42 %, which corresponds to the results obtained in the study of Hulleman et al. (1994) where a crystallinity of 40- 48 % was calculated for the cellulose from cotton. Crystallinity of the cell wall of maize is much higher (58 %) than in the cell wall of spruce and maple. Similar crystallinity value was reported in maize by Ragauskas et al. (2015) – 50 %. This higher crystallinity in maize may be due to better packing and more regular distribution of cellulose microfibrils, as was observed on the FDLD images (Fig. 4) and shown by narrower LD distribution comparing with the woody species. The crystallinity results are in agreement with the FTIR spectral data (Figs. 1 and 2, Table 12). The A_{1375}/A_{2900} ratio of band intensities in the FTIR spectra, as a measure of sample crystallinity (Fan et al. 2012; Nelson and O'Connor 1964; O'Connor et al. 1958), was highest for maize (1.5) and lower for maple and spruce (0.7 and 0.6 for maple and spruce respectively). Previous studies (Fan et al. 2012; Popescu et al. 2007, 2011; Yuan et al. 2013; Zhao 2013) have shown two characteristic spectral regions for intra-molecular hydrogen bonds, one region for inter-molecular bonds, and one region characteristic for the free OH. In order to differentiate the hydrogen bond type, Gaussian deconvolution model was applied in the OH spectral region (Fig. 2, Table 1). Results of deconvolution of OH region in FTIR spectra show fine differences among the three species in OH content related to inter- and intra-molecular hydrogen bond. The highest percent of intra-molecular hydrogen bonds in maize may reflect more compact structure of cellulose in this species, which is in accordance with the highest crystallinity (Fig. 3, Table 2), the highest A_{1375}/A_{2900} ratio (Fig. 1) and FDLD image and LD distribution (Fig. 4). The lowest percent of inter-molecular hydrogen bonds in maize cell wall indicates weaker interactions between cellulose and lignin. One can propose that such structural organization may enable simpler deconstruction of this cell wall.

It is known that different hemicelluloses, like glucomannan in softwood, xylan in hardwood and glucuronoarabinoxyilan in maize, are associated with cellulose (Akerholm and Salmen 2001; Dammstrom et al. 2009; Ruel and Joseleau 2005), and thus may contribute to crystallinity of the whole cell wall structure. Fluorescence of the cell wall originates from lignin macromolecule (Albinsson et al. 1999; Olmsted and Grey 1993). By using fluorescence spectroscopy and spectral deconvolution we monitored the complexity of the structure of lignin and its different networking with polysaccharides in the cell wall structures. Figure 5 shows that it is necessary to choose the appropriate number of components in the mathematical analysis for each plant species, depending on the complexity of the cell wall structure. Overlay of APDs shows clear difference in lignin structure. The spectral components obtained as result of spectral deconvolution in the form of an approximation of the probability density – APD (Djikanović et al. 2012a,b; Donaldson et al. 2010; Kalauzi et al. 2007; Radotić et al. 2006), are designated I – V (Fig. 6). The spectra of maize cell walls have three APD peaks (Fig. 6, solid lines)

indicating that their spectra consist of three components, each corresponding to an independent fluorophore, designated II–IV.

Such simpler lignin structure may contribute to more ordered packing of cellulose, which may lead to higher crystallinity of this sample. These differences are in accordance with the simplest maize cell wall structure obtained on DP-LSM image (Fig. 4). Analysis of fluorescence spectra shows more complex structure in the woody species (Fig. 6). The spruce cell walls contain five components corresponding to independent emitting structures (designated I – V), while maple sample contains four emitting structures (designated I – IV). The blue shift of the component IV at 480 nm of the maize cell wall comparing with the other two samples may be result of the rigidity of fluorophore environment which reduces the possibility of a non-fluorescent radiation relaxation in the ground electronic state. It was previously shown that long-wavelength spectral components in lignin spectrum correspond to the extended domains consisting of conjugated bonds (alternating C-C and C=C bonds) within this macromolecule (Djikanovic et al. 2012; Donaldson et al 2010). The red shift of the component V in the APD of spruce lignin comparing to the maple sample (Fig. 6) indicates larger conjugated domain in spruce. In comparison to hardwood (GS-lignin), softwood (G-lignin) has a lower amount of methoxy groups which reduces steric effects and increases probability of the occurrence of a conjugated structure. Due to the presence of greater amounts of methoxy groups in syringyl and guaiacyl units, it may be a higher possibility of cross-linking among the macromolecule chains and a lower possibility for the occurrence of conjugated structures. Variations in component positions observed in the three samples may be a consequence of presence of different hemicelluloses in these plant species (Fry 1986; Newman 1992). This may influence lignin-carbohydrate complexes (LCC). For example it is already known that hemicelluloses influence lignin networking in the cellulose macromolecule (Burlat et al. 2000; Ruel et al. 1978, 1979). A variety of hemicelluloses will result in different networking of lignin and cellulose reflected in the whole arrangement of cell wall (Anterola et al. 2002; Ha et al. 2002).

The approaches that have not been applied before in this kind of study, namely FDLD (Fig. 4) and fluorescence spectroscopy combined with spectral deconvolution (Figs. 5 and 6), provided strong evidences of simpler cell wall organization in the maize sample than in both woody species. The isolated maize cell walls have a great amount of electrostatic interactions and high content of hydrogen bonds that lead to more regular packing of cellulose molecules, resulting in a simpler structure, with higher crystallinity and higher linear order of the cell wall in relation to the walls of the samples of woody plants. This, together with economic aspects (high amount of maize waist in this region), makes this cell wall more suitable as a main source of biomass. The DP-LSM method is shown to be a simple and rapid way to check the structural complexity in the cell wall, through cellulose labeling. Complementary screening of the structural complexity is obtained by using fluorescence spectroscopy, following lignin genuine emission in the samples. These data, together with XRD and FTIR method including deconvolution of hydrogen bond region, provide a better insight in whole arrangement of the cell wall.

Conclusions

This research focuses on the study of interactions between macromolecules in the cell walls of different plant origin. It was shown that the isolated cell walls of maize have higher content of hydrogen bonds that lead to more regular packing of cellulose molecules and simpler lignin structure, resulting in higher crystallinity of the cell wall in relation to the walls of the sample of woody plants. We have also shown that FDLD and fluorescence spectroscopy with spectral deconvolution may be efficient methods for the first screening of structural complexity of the cell wall samples.

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Fig. 1. FTIR spectra of three samples of isolated cell walls. a) FTIR region from 1800 cm^{-1}- 700 cm^{-1} and b) FTIR region from 3800 cm^{-1}- 2600 cm^{-1} (area-normalized spectra). Maize, spruce and maple cell walls are designated by solid, dashed and dot line, respectively.

Fig. 2. Deconvolution of hydrogen bond region of FTIR spectra from three different plant species, by using Gaussian model: maple-upper panel, spruce-middle panel and maize-lower panel

Fig. 3. Diffraction patterns of the cell walls isolated from the maple branch (left panel), the branches of a spruce (middle panel) and maize stem (right panel) after correction for baseline and amorphous parts of diffractogram

Fig. 4. Confocal fluorescence intensity (upper panel), confocal fluorescence detected linear dichroism (FDLD) measurements (middle panel) and linear dichroism distributions (lower panel) of the isolated cell walls of maize (a, d, g), maple (b, e, h) and spruce (c, f, i) stained with Congo Red. The blue and yellow regions in FDLD images are for the horizontal and for the vertical dipole orientations, respectively.

Fig. 5. Deconvolution of the fluorescence emission spectra of the cell walls recorded after excitation at 370 nm, by using Log-normal model: a) overlay of area-normalized spectra of spruce, maple and maize sample, b) Five-component deconvolution of fluorescence spectra isolated from cell wall of maple branch, c) Four-component analysis of fluorescent spectra of isolated cell wall from spruce d) Three-component analysis of isolated cell wall from maize stem. Maize, spruce and maple cell walls are designated by solid, dashed and dot line, respectively. Gaussian components are presented by thin solid lines.

Fig. 6. Overlay results of approximate distribution of the probability density (APD) for spectral components position, for three cell wall samples. Spectral components are indicated by numbers I to V. Maize, spruce and maple cell walls are designated by solid, dashed and dot line, respectively.

Fig. 4_greyscale. Confocal fluorescence intensity (upper panel), confocal fluorescence detected linear dichroism (FDLD) measurements (middle panel) and linear dichroism distributions (lower panel) of the isolated cell walls of maize (a, d, g), maple (b, e, h) and spruce (c, f, i) stained with Congo Red. The dark-grey and light-grey regions in FDLD images are for the horizontal and for the vertical dipole orientations, respectively.