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Distribution of lignin and cellulose in compression wood tracheids of *Pinus yunnanensis* determined by fluorescence microscopy and confocal Raman microscopy

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ABSTRACT

A detailed understanding of the topochemistry of compression wood tracheid walls is important from the perspectives of plant biochemistry and commercial utilization. The present study aimed to determine the anatomy and distribution of lignin and cellulose in situ in compression wood tracheids of *Pinus yunnanensis* by fluorescence microscopy and confocal Raman microscopy. Anatomical observation by fluorescence microscopy revealed that *P. yunnanensis* can be classified as mild compression wood and the lignin distribution in tracheid walls was heterogeneous. Confocal Raman microscopy was used to examine the distribution of lignin and cellulose within morphologically distinct tracheid wall regions. The histochemical observations indicated that the highest level of lignification occured in the cell corners middle lamella (CCML) some what less in the outer S2 layer ($S2_L$), while the lowest lignin concentration was found in the S2. In contrast, cellulose distribution showed the opposite pattern with the highest cellulose concentration occurring in the S2 layer. The Raman analysis also indicated that the cellulose microfibril angle (MFA) in the S1 was high and thus the cellulose fibrils and the C—C, C—O stretching occur at a large angle.

1. Introduction

Currently, the general trends toward sustainability in the forest industry and growing interests in bioenergy have promoted the utilization of wood components as natural resources. Plant cell walls are the most abundant renewable resource of photosynthetic carbon (Herrera, 2006; Himmel et al., 2007; Ragauskas et al., 2006; Savage et al., 2008; Sun et al., 2011). They have a heterogeneous and complex structure, primarily consisting of cellulose, hemicelluloses, and lignin. Cellulose, made up of (1 → 4)-β-D-glucopyranose units, is abundant in the nature. Crystals of cellulose form microfibrils which are embedded in matrix polymer and represent the major scaffold of the cell wall. Hemicelluloses are composed of different 5- and 6-carbon monosaccharide units and link cellulose microfibrils into fibers and cross-link with lignin. Lignin, after cellulose and hemicelluloses, is the third most abundant natural polymer, which is commonly defined as a complex and irregular poly-phenylpropanoid heteropolymer derived from oxidative polymerization with three major structural units: guaiacyl, syringyl and *p*-hydroxyphenyl units (Boerjan et al., 2003; Dixon and Srinivasa Reddy, 2003). Previous studies have shown that the plant cell wall components are not uniformly distributed within morphologically distinct cell wall regions (Schmidt et al., 2009; Williams et al., 1996). Additionally, the effective use of plants for industrial purposes is in fact largely dependent on the extent of lignification. For example, in biomass conversion for biofuels, lignin inhibits saccharification aimed at producing simple sugars for fermentation to ethanol (Ralph et al., 2007). In contrast, the presence of lignin in a high concentration within the cell walls is regarded as a positive benefit in the dry and wet formed fiberboard industry. Therefore, it is of vital importance to know the influence of structural effects on the mechanical, chemical, and biological behaviors of plant materials (Olsson et al., 2011).

It has been reported that numerous techniques, such as nuclear magnetic resonance spectroscopy (Kim and Ralph, 2010), chromatography (Fagard et al., 2000), and mass spectrometry (Gholipour et al., 2008) have been used to analyze plant cell wall components. Such methods are disruptive involving destruction of native-state structures, isolation of components from plant cell walls, or extensive chemical treatment of plant cell walls. Therefore, the ability to generate images of the chemical composition from plant cell wall nondestructively would be a significant advance.

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Raman microscopy was shown to be well suited to give insights into both the composition and structure of lignocellulosic materials in situ (Atalla and Agarwal, 1986; Bond and Atalla, 1999; Sene et al., 1994). Advanced confocal Raman microscopy, with the advantages of high resolution and simple operation, has been widely used to investigate the spatial distribution and orientation of cell wall polymers on the micron level in a close to native state, in particular cellulose and lignin (Edwards et al., 1997; Fischer et al., 2005; Gierlinger et al., 2010; Himmelsbach et al., 1999; Morrison et al., 2003; Peetla et al., 2006; Schenzel et al., 2009).

Pinus yunnansis, a conifer species widely distributed in the Yunnan plateau in China, approximately accounts for 70% of the forestry biomass in this region. The pollen, resin and cones are all used in cosmetics and drugs (Xue et al., 2012). As a potential biomass resource, much attention has been paid to the applications in papermaking and bio-ethanol production (Dai, 2006). However, the utilization efficiency of this renewable species is highly affected by its structural properties, such as the relative polymer content and accessibility, the thickness of cell wall, and distribution of the main components (cellulose and lignin). It is worth noting that the species generally exists in the form of compression wood, in which the lignin and cellulose distribution is distinguished from normal wood.

The aim of the present study was to investigate the severity of the *P. yunnanensis* compression wood and the distribution of lignin and cellulose. The morphological features of samples and lignin distribution in tracheid walls were examined by fluorescence microscopy. Confocal Raman microscopy was used to acquire chemical images and spectra of tracheid wall components (lignin and cellulose) in different wall layers simultaneously. Differences in lignin and cellulose concentration, localization, and orientation in the tracheid walls were visualized without staining or labeling of the tissue.

2. Materials and methods

2.1. Materials

Samples were cut from *P. yunnanensis* tree grown at the botanical garden of Southwest Forestry University, China. A wood disc about 5 cm thickness containing 20 growth rings was collected at a height of 3 m from the leaning stem (10–15°). Compression wood samples were collected from the fifth growth rings on the lower side and cut into small blocks 2 × 1 cm preserved in glycerin/ethanol (1:1, v/v) under vacuum.

2.2. Fluorescence microscopy

For fluorescence microscopy, transverse sections of 5 μm thickness were prepared with a sliding microtome. After dehydration through an ethanol series (50%, 70%, 90% and 100%), the sections were mounted in glycerol and covered with a coverslip (0.17 mm thickness). Sections were examined with a Leica fluorescence microscope (DM6000 B) using an ultra-pressure mercury lamp for illumination. The excitation wavelength was 435–480 nm and the emission wavelength at 495–600 nm was used for imaging lignin autofluorescence.

2.3. Confocal Raman microscopy

The transverse sections (5 μm) were placed on a glass slide with a drop of water, and then covered with a coverslip (0.17 mm thickness) for Raman detection. The biochemical components within *P. yunnanensis* tracheid walls were examined in the native state using confocal Raman microscopy. Data was acquired by using a LabRam Xplora exquisite full-automatic confocal Raman microscope (Horiba Jobin Yvon) equipped with a confocal microscope (Olympus BX51), a motorized *x*, *y* axis, and a high numerical aperture (NA) microscopy objective from Olympus (MPlan 100× objective, NA = 1.40). The Raman light detected with an air cooled back-illuminated CCD. A linear-polarized green laser with $\lambda = 532$ nm was focused with a diffraction-limited spot size ($0.61\lambda/NA$). The laser power on the sample was about 8 mW. For mapping, an integration time of 4 s and steps of 0.6 μm were applied and every pixel corresponded to one scan. For acquiring spectra, each location was obtained by averaging 2 s cycles. The wavenumber ranged from 3200 to 600 cm^{-1} with a confoal aperture at 400 μm and slit width at 100 μm. The reported depth resolution for the 400 μm confocal hole, based on the silicon (standard) phonon band at 520.7 cm^{-1}, was 2 μm. The lateral resolution of the confocal Raman microscope was 1 μm, which was significantly lower than the theoretical prediction ($0.61\lambda/NA \approx 232$ nm).

For the measurement setup, imaging processing and spectral analysis, Labspect 5 software was used. The overview chemical images enabled separation of cell wall layers differing in chemical composition and to mark defined distinct cell wall areas for constructing average spectra from regions of interest (such as CCML, S2 and $S2_L$) for a detailed analysis. The spectra were baseline corrected with the Savitsky–Golay algorithm for spectroscopic analysis.

3. Results and discussion

3.1. Fluorescence microscopy analysis

Compression wood, commonly found on the lower side of branches and leaning stems is known to be classified from mild to severe wood based on its anatomy (Shelbourne and Ritchie, 1968). For severe compression wood, a rounded cell outline with intercellular spaces at the corners, a thick secondary wall containing helical cavities, excessive lignification particularly in the outer S2 region ($S2_L$), and the absence of S3 layer are all the obvious features (Yumoto et al., 1982). However, mild compression wood is characterized by many different partial combinations of the complete set of compression wood features described above, forming a continuum between normal and severe compression wood. The predominant feature of mild compression wood is an increased degree of lignification in the $S2_L$ especially adjacent to the corners. The cell circularity and the presence of the S3 layer and intercellular spaces are variable in mild compression wood (Donaldson et al., 1999; Singh and Donaldson, 1999).

The sample examined was found to be anatomically distinct in terms of compression wood features. As shown in Fig. 1, the cell walls of compression wood tracheids in *P. yunnanensis* were thicker than those of normal wood (the data not shown). The well-ordered tracheids with thick cell wall showed some degree of rounding. A distinct $S2_L$ layer separated from the secondary wall with a discontinuous profile was thickest at the cell corner middle lamella (CCML). Neither helical cavity in the secondary wall nor intercellular space was observed. Based on the microscopic features above, *P. yunnanensis* can be classed as mild compression wood. The anatomical features can influence wood physical properties. It has been reported that the thick tracheids with rounded appearances of compression wood induce increased density and reduced permeability that influence the effectiveness of pretreatment (Diaz-vaz et al., 2009; Torr et al., 2012). The analogous anatomical characterizations are observed in *P. yunnanensis* compression wood tracheids, which are associated to unsuitable properties for many of the products made from this wood, for example: pulp and paper and biochemicals.