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# Enhanced cellulose orientation analysis in complex model plant tissues

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ABSTRACT

The orientation distribution of cellulose microfibrils in the plant cell wall is a key parameter for understanding anisotropic plant growth and mechanical behavior. However, precisely visualizing cellulose orientation in the plant cell wall has ever been a challenge due to the small size of the cellulose microfibrils and the complex network of polymers in the plant cell wall. X-ray diffraction is one of the most frequently used methods for analyzing cellulose orientation in single cells and plant tissues, but the interpretation of the diffraction images is complex. Traditionally, circular or square cells and Gaussian orientation of the cellulose microfibrils have been assumed to elucidate cellulose orientation from the diffraction images. However, the complex tissue structures of common model plant systems such as Arabidopsis or aspen (*Populus*) require a more sophisticated approach. We present an evaluation procedure which takes into account the precise cell geometry and is able to deal with complex microfibril orientation distributions. The evaluation procedure reveals the entire orientation distribution of the cellulose microfibrils, reflecting different orientations within the multi-layered cell wall. By analyzing aspen wood and Arabidopsis stems we demonstrate the versatility of this method and show that simplifying assumptions on geometry and orientation distributions can lead to errors in the calculated microfibril orientation pattern. The simulation routine is intended to be used as a valuable tool for nanostructural analysis of plant cell walls and is freely available from the authors on request.



## 1. Introduction

The arrangement of cellulose microfibrils in the plant cell wall plays a crucial role in anisotropic plant growth and mechanical performance of tissues (Baskin, 2005; Cave, 1969; Reiterer et al., 1999; Yamamoto et al., 2001). Hence, knowing the microfibril orientation in the cell wall is a prerequisite for understanding growth patterns and mechanics of plants. In addition, knowledge of cellulose orientation is important for appropriate manufacturing and use of wood and wood related products in construction and other technical applications. The small size of the cellulose microfibrils and the complex structure and chemistry of cell walls makes it challenging to directly observe the microfibrils in the native plant cell wall. Several methods, all with specific potentials and limits, have been developed for evaluating cellulose orientation in plant tissues. For an overview of the different methods available we refer to the review of Barnett and Bonham (2004).

Wide angle X-ray diffraction (WAXD) and small angle X-ray scattering (SAXS) are among the most frequently used methods for elucidating cellulose orientation because the orientation can be resolved for samples in their native state, whereas most other methods involve extensive sample preparation. In addition, valuable structural information on cellulose is obtained. However, the interpretation of the diffraction pattern is complex for the following reasons: (i) diffraction patterns obtained on the detector are not only influenced by the orientation distribution of the cellulose microfibrils, but also by the geometry of the cells; (ii) single peaks of the diffraction pattern of plant cell walls are broad because of the small size of the cellulose crystallites within the cellulose microfibrils; (iii) peak overlapping occurs because the diffraction pattern represents the sum of different microfibril orientation distributions within a multi-layered cell wall.

Assuming either regular cell geometry (flat, circular or square cells) and/or Gaussian distribution of the cellulose microfibrils, several procedures exist for evaluating cellulose orientation from the WAXD patterns in a simple and rapid way (Cave, 1966; Evans, 1999; Lichtenegger, 1998; Meylan, 1967; Saren and Serimaa, 2006; Saren et al., 2001). But often cell geometries and

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cellulose orientation distributions are more complex, e.g. in xylem tissues of model plants such as *Arabidopsis thaliana* and *Populus sp*. In these cases alternative evaluation procedures are required for accurate calculations. Moreover, when tissues with different cell geometries and different microfibril distributions are compared (for instance when comparing mutant plants with wildtype plants) it is important to obtain the complete microfibril orientation distribution. It is further necessary to separate changes in the diffraction pattern caused by changes in the orientation of cellulose microfibrils from those caused by alterations in cell geometry and ratios of cell-wall layer thicknesses. Accordingly, Entwistle et al. (2007) and Entwistle and Terrill (2000) developed a simulation routine which takes into account the precise cell geometry, but this procedure is restricted to a Gaussian distribution of the microfibrils.

Here we present a method which not only incorporates the precise cell geometry of the sample but also the multi-layered structure of the cell wall with potentially different cellulose orientation distributions. The output is the complete orientation distribution of the cellulose microfibrils in the investigated sample volume. The principles and versatility of the procedure are demonstrated by determining the microfibril orientation distribution in the xylem of *Populus tremula* (aspen) and stems of *Arabidopsis thaliana*. This methodical upgrade will serve the growing interest in ultrastructural analysis of plant cell walls in heterogeneous tissues and genetically modified species (Mellerowicz and Sundberg, 2008).

## 2. Materials and methods

### 2.1. Simulation approach for analyzing wide angle X-ray diffraction patterns

The present analysis uses a WAXD pattern of the cellulose in the plant cell wall and a cross-section of the irradiated plant tissue (Fig. 1a–c) to elucidate the cellulose microfibril orientation distribution in the plant cell wall. From the diffraction pattern, an azimuthal intensity profile (intensity vs. azimuthal angle, i.e. orientation) of the most intense Bragg peak of the crystalline cellulose (the (200)-peak) is generated (Fig. 1b). The cross-sectional image is needed to obtain the corresponding cell geometry in terms of cell wall orientation and length (Fig. 1d). As parameter to describe the cellulose orientation distribution the microfibril angle (MFA), which is the angle between the long axis of a cellulose microfibril and the long axis of the cell, is used. Taking the general reflection condition for the (200)-peak of cellulose (Cave, 1966), which relates the azimuthal angle on the detector to the microfibril angle in the plant cell wall for a given cell wall orientation an azimuthal intensity profile is calculated for the sample for a uniform orientation distribution of the cellulose microfibrils. By introducing appropriate distribution functions for the orientation of the cellulose microfibrils, such as a Gauss or a Lorentz function or a sum of several distribution functions, the calculated azimuthal intensity profile is fitted to the measured azimuthal intensity profile (Fig. 1e). In case of a Gauss function the relevant fit parameters would be the mean $\mu$, its standard deviation $\sigma$ and the amplitude. The microfibril orientation distribution is then obtained in terms of the relative contribution of individual microfibril angles in the range of 0–90°. The mean of the Gaussian distribution would then represent a mean microfibril angle (Fig. 1f).

### 2.2. Plant material

#### 2.2.1. Populus tremula

A longitudinal-radial section of 40 μm thickness and 33 mm width was cut from a wood disk of a 30 year old aspen *(P. tremula)* with a rotary microtome in wet condition. The tree was grown in the field outside Umea, Sweden. The section was air dried for the X-ray measurements.

#### 2.2.2. Arabidopsis thaliana

Wildtype plants of the ecotype Col-0 were germinated and grown following the procedure of Persson et al. (2005). The germination was on standard MS medium under continuous light (140–220 mol $m^{-2}$ $s^{-1}$) at 23 °C. Seedlings were transferred to soil and grown in greenhouse chambers under 16 h light/8 h dark conditions at 23 °C and harvested at the age of three weeks. Short pieces of two freeze-dried stems were measured.

### 2.3. X-ray diffraction measurement

#### 2.3.1. X-ray diffraction on poplar xylem tissue using synchrotron radiation

The $\mu$-Spot Beamline at the synchrotron facility BESSY II, Berlin, Germany (Paris et al., 2007) was used for scanning synchrotron WAXD measurements on *P. tremula*. The X-ray beam diameter and the step width of the scan were set to 150 μm. The radiation energy was set to 15 keV corresponding to a wavelength of 0.08265 nm. The (200)-Bragg peaks of cellulose which were taken for orientation analysis occurred at a scattering angle $2\theta$ of 11.8°. The sample-detector-distance was set to 371.6 mm and the exposure time was set to 30 s for each frame. The wood section was scanned from pith to cambium in ambient air. The longitudinal axis of the cells was oriented vertically and the section was orientated perpendicular to the incident beam. Fig. 2 shows the schematic experimental setup for a single cell wall with the relevant coordinate system, axes, angles and vectors.

#### 2.3.2. X-ray diffraction on Arabidopsis stems using a lab source

A Bruker Nanostar (Bruker AXS, Karlsruhe, Germany) with a HiStar area detector was used as lab source for measurements on *Arabidopsis* stems. Cu K$\alpha$ radiation with a wavelength of 0.154 nm (8 keV) was used with the (200)-peaks of cellulose appearing at a scattering angle $2\theta$ of 21°. The beam diameter was approximately 400 μm and the sample-detector distance 68 mm. The samples were measured in an evacuated chamber with the long axis of the stem being perpendicular to the incident X-ray beam. This is the same geometrical configuration as described for the measurements of aspen.

#### 2.3.3. Generating azimuthal profiles of the X-ray diffraction patterns

Azimuthal intensity profiles of the diffraction patterns were obtained by radially integrating the intensity of the (200)-peaks of cellulose within $2\theta \pm 0.2°$ with an azimuthal step size of 1°.

### 2.4. Detailed simulation and fitting procedure

#### 2.4.1. Calculating the azimuthal position of the (200)-peaks of cellulose

The simulation procedure is based on the general reflection condition for the (200)-peaks of cellulose. It relates the azimuthal angle $\varphi$ of the (200)-Bragg peak to the microfibril angle of the irradiated cell wall for a given cell wall orientation. The reflection condition was initially derived by Cave (1966). Using the notation of Fig. 2, Eq. (1a) and (b) are obtained for the front wall and the back wall respectively. The detailed mathematics is described in the Appendix A.

$$-\tan\theta \cdot \sin\alpha + \cos\alpha \cdot \sin\varphi + \cos\varphi \cdot \cot\nu = 0 \quad (1a)$$

$$\tan\theta \cdot \sin\alpha - \cos\alpha \cdot \sin\varphi + \cos\varphi \cdot \cot\nu = 0 \quad (1b)$$

Solving these equations for the azimuthal angle $\varphi$ leads to two equations (Eqs. (A.5a) and (A.5b), Appendix A) which give the azimuthal position of the four Bragg peaks $\varphi_{11}$–$\varphi_{22}$ on the