UTILIZATION OF SOFT-ROT CAVITY ORIENTATION FOR THE DETERMINATION OF MICROFIBRIL ANGLE. PART I

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ABSTRACT

These studies utilize the decay cavities formed by the soft-rot fungus, *Phialocephala dimorphospora,* to determine the orientation of the cellulose fibrils in the cell wall. In this study, the microfibril angle was determined utilizing three methods: X-ray diffraction (T method), iodine staining, and orientation of the soft-rot cavities. The results demonstrate good agreement between the three techniques and verify that the decay cavities are formed in a direction parallel to the cellulose microfibrils and therefore can be used to determine the orientation of the cellulose microfibrils. One advantage of the soft-rot method over the X-ray method is the ability to measure angles of any size, including those of juvenile wood.

Keywords: Cellulose, fibers, iodine crystal deposition, microfibril angle, S2 angle, soft-rot cavities, tracheids, X-ray diffraction.

INTRODUCTION

A reliable technique that utilizes the elongated cavities formed by soft-rot decay is being developed to measure the microfibril angle. By depositing iodine crystals in a tracheid containing soft-rot cavities, it was shown that soft-rot cavities lie parallel to the cellulose microfibrils (Bailey and Vestal 1937). That soft-rot cavities are oriented parallel to the microfibrils has also been demonstrated with scanning electron microscopy (Crossley and Levy 1977) and transmission electron microscopy (Levi and Preston 1965; Hale and Eaton 1983).

Soft-rot cavities form in the S2 cell-wall layer by a 2-phase, oscillatory growth pattern (Hale and Eaton 1985). Attack on the wood cell wall begins with a hypha in the lumen boring horizontally through the wall. Within the S2 layer, the hypha branches approximately 90° in one or two directions, forming an "L" or "T" branch. Following T- or L-branching within the S2 layer, the first phase, hyphal extension, occurs. It is during this extension phase of cavity formation that the proboscis hypha grows parallel to the microfibrils. The extension phase then stops and the second

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FIG. 1. Soft-rot cavities in a tracheid from a maceration prepared from a decayed block of southern pine. The cavities follow the microfibril angle in the S2 layer of the cell wall. Soft-rot cavities are also visible in a pit border forming a circular chain of cavities. 500×.

FIG. 2. The soft-rot cavity method of determining microfibril angle. Utilizing the image analysis software, the longitudinal axes of the tracheids are aligned to an angle of 0° with a rotating microscope stage. A line is drawn parallel

phase, cavity widening, occurs. During cavity widening, the sides of the enlarging cavity remain parallel to the original proboscis hypha. The formation of soft-rot cavities in delignified fibers (Nilsson 1974) indicates that cavity shape is dependent on cellulose structure, a finding that supports the idea that cavity formation follows the hydrolysis planes in the cellulose crystal lattice (Wardrop and Jutte 1968; Jutte and Wardrop 1970). Examination of soft-rot cavities caused by many fungi shows that cavity shape is more a reflection of the structure of the cell wall than a function of fungus species (Courtois 1963; Anagnost 1998). The extension and widening phases repeat, forming chains of cavities. When formed, these cavities are relatively large and easily visualized with either polarized light or Nomarski DIC (differential interference contrast) microscopy (Fig. 1).

The objectives of this research were to utilize the fact that the soft-rot cavities are large, easily visualized, and parallel to the microfibrils to: 1) develop a method for determining microfibril angle for both thin sections and fibers, and 2) validate this method through a comparison with a direct method (iodine staining) and an indirect method (X-ray diffraction).

Part 2 is a continuation of this study that relates the orientation of the soft-rot cavities, and thus the microfibrils, to the various hypotheses of cell-wall structure, with particular attention to their orientation at the ends of tracheids (Anagnost et al. 1999).

METHODS AND MATERIALS

Cross-sectional disks of loblolly pine (*Pinus taeda* L.) were obtained from wood harvested in eastern Maryland. They were wrapped and transported to Syracuse and immediately frozen upon arrival. Two disks were cut, one 20.2 cm in diameter with 50 growth rings, the other 19.4 cm in diameter with 46 growth rings.

From the disks, blocks were cut along a radius at regular intervals from the seventh to the fiftieth ring, including both juvenile and mature wood. A total of 22 blocks (11 earlywood and 11 latewood) were cut utilizing the same dimensions (3 cm (L) × 1 cm (T) × 0.15 cm (R)) used by Meylan (1967) to study microfibril angle. The final tangential slice (radial thickness dimension) was cut with a sliding microtome.

X-ray diffraction of blocks

First, the blocks were analyzed with a Rigaku X-ray diffractometer. Microfibril angle was calculated as 0.6T (Meylan 1967).

After X-ray analysis, thin sections were prepared from each block (Meylan 1967). Blocks were boiled for one hour, air-dried, glued to support blocks with epoxy, and sectioned with a sliding microtome after soaking. Sections 20 μm thick were cut from each block. The sections (3 cm (L) × 1 cm (T) × 0.0020 cm (R)) were sliced in half along the tangential plane (final dimensions 1.5 cm (L) × 1 cm (T) × 0.0020 cm (R)). One-half was analyzed using the soft-rot method (see below) while the other half was analyzed with the iodine staining method (Senft and Bendtson 1985), thus providing matching samples. For the soft-rot and iodine methods, 2 sections were examined from each X-ray block.

Soft-rot cavity method

The thin sections were soaked in reduced salts nutrient solution (RSNS) consisting of

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to the cavity at the midpoint of the cell, and the microfibril angle is automatically measured with the image analysis software (850× magnification).

FIG. 3. The iodine crystal deposition method of determining microfibril angle. Utilizing the image analysis software, the longitudinal axes of the tracheids are aligned to an angle of 0° with a rotating microscope stage. The dark lines are iodine crystals that formed within splits between microfibrils, following the microfibril angle. A line is drawn parallel to the iodine crystals at a point equal distances from the longitudinal walls, and the microfibril angle is automatically measured with the image analysis software (850× magnification).

1.5 g NH_4NO_3, 2.5 g KH_2PO_4, 2 g K_2HPO_4, 1 g $MgSO_4*7H_2O$, and 2.5 g glucose per liter (Worrall et al. 1991), autoclaved (250°C; 103 kPa (15 psi)), placed on sterile glass microscope slides, and covered with a sterile cover slip. The slides were placed on plastic mesh rectangles in Petri dishes (2.0% malt extract agar) previously inoculated with *Phialocephala dimorphospora* Kendrick P109 (Anagnost et al. 1994). A small piece of inoculum was transferred to the edge of the section to accelerate decay. The slide cultures were incubated at 26°C. After 6 to 14 weeks, the slides were removed from Petri dishes, sections were mounted in glycerin and examined with Nomarski DIC microscopy. Growth rate of the slide cultures varied widely. The initiation of fungal attack in the form of soft-rot cavities appeared to be sensitive to both high and low moisture contents. Additional sterile RSNS was added to the sections if additional moisture was needed. Sterile filter paper was applied to remove excess moisture.

Microfibril angle measurements using soft-rot cavity orientation

Soft-rot cavities were observed in unstained sections using Nomarski DIC microscopy. Microfibril angles were measured using image analysis software (Universal Imaging). Using a microscope with a rotating stage, the longitudinal axis of each tracheid was aligned to the 0° reference line. The angle was measured by drawing a line parallel to the portion of each soft-rot cavity, which was equidistant from the radial walls (Fig. 2). For each slide consisting of one earlywood or latewood section, one scan was made, counting one to several cavities per tracheid across the slide until 50 cavities were examined. Two sections were examined for each sample, for a total of 100 cavities per sample.

Iodine crystal deposition method

Sections were dried and rewetted to induce splitting along the microfibrils. The sections were placed on glass microscope slides and treated according to Senft and Bendtsen (1985) to produce iodine crystals. The sections were stored at 50% ethanol, if necessary, dehydrated in an alcohol series, and rinsed with absolute alcohol 3 times. A solution of 1% iodine-potassium iodide (1 g I; 1 g KI in 100 ml distilled H_2O) was applied for 2–10 seconds. The excess solution was removed and 2 drops of 60% nitric acid were applied to the sections. Cover slips were applied and the sections were examined immediately.

Microfibril angle measurements using iodine crystals

The angle of iodine crystals to the longitudinal axis of each tracheid was determined with the same image analysis procedure utilized in the soft-rot cavity method except that the sections were examined in brightfield. The focal plane was adjusted to the plane of the S2 layer by focusing through the wall (Fig. 3).

Sample size determination and statistical analysis

In order to determine a sample size with an acceptable standard deviation, preliminary measurements were performed on a radial section of southern pine containing soft-rot cavities. The results indicated that for combined earlywood and latewood, a sample size of 30 soft-rot cavities would yield 95% C.I. of ± 3.1, a sample size of 50 would yield a 95% confidence interval of ± 2.4, and a sample size of 100 would yield a 95% C.I. of ± 1.7. Separating earlywood and latewood lowered the standard deviation and decreased the sample size needed to achieve acceptable variability. For earlywood alone, a sample size of 50 would yield a 95% C.I. of ± 1.80; a sample size of 100 would yield a 95% C.I. of ± 1.3. For latewood, a sample size of 50 would yield a 95% C.I. of ± 0.9; a sample size of 100 would yield a 95% C.I. of ± 0.7.

Comparison of X-ray analysis of blocks to direct measurements on thin sections

For each X-ray block, 100 soft-rot cavities or iodine-stained splits were observed, 50

from each section. According to the data obtained in the preliminary study of soft-rot orientation, a sample size of 100 should yield 95% confidence intervals of ± 0.7 for latewood and ± 1.3 for earlywood.

RESULTS AND DISCUSSION

The relationship between the microfibril angles obtained with the soft-rot method and those obtained with the iodine method was determined by a regression analysis (Fig. 4a). The regression line shows a very close correlation between the soft-rot and iodine methods with a slope of 1.000 and r^2 of 0.88 (correlation coefficient (r) of 0.94). The relationship between the microfibril angle as determined by the soft-rot method and the results obtained with X-ray diffraction was also compared with a regression analysis (Fig. 4b). Again, there was close correlation with a slope of 1.24, r^2 of 0.94, and correlation coefficient (r) of 0.97. A regression analysis comparing the X-ray method to the iodine method indicated again a close correlation (Fig. 4c) with a slope of 0.80, r^2 of 0.89 and correlation (r) of 0.94. These results are quite similar to other recent studies (Kretschmann et al. 1998; Huang et al. 1998; Meylan 1967) which compared the results of microfibril angle determination obtained by X-ray diffraction and iodine staining.

The close correlation with both methods of iodine staining and X-ray diffraction demonstrates that the soft-rot cavity method is an accurate and viable method of determining the microfibril angle. For the two direct methods of determining microfibril angle, soft-rot cavity and iodine, the sample size utilized (100) resulted in a low amount of variability. Ninety-five percent confidence intervals ($\alpha = 0.05$) ranged from 0.8 to 2.2 with the iodine method and 0.8 to 2.8 with the soft-rot method. This indicates that a smaller sample size would probably be sufficient for analysis.

The variability obtained for earlywood samples was greater than that obtained for latewood samples. For example, for the soft-rot

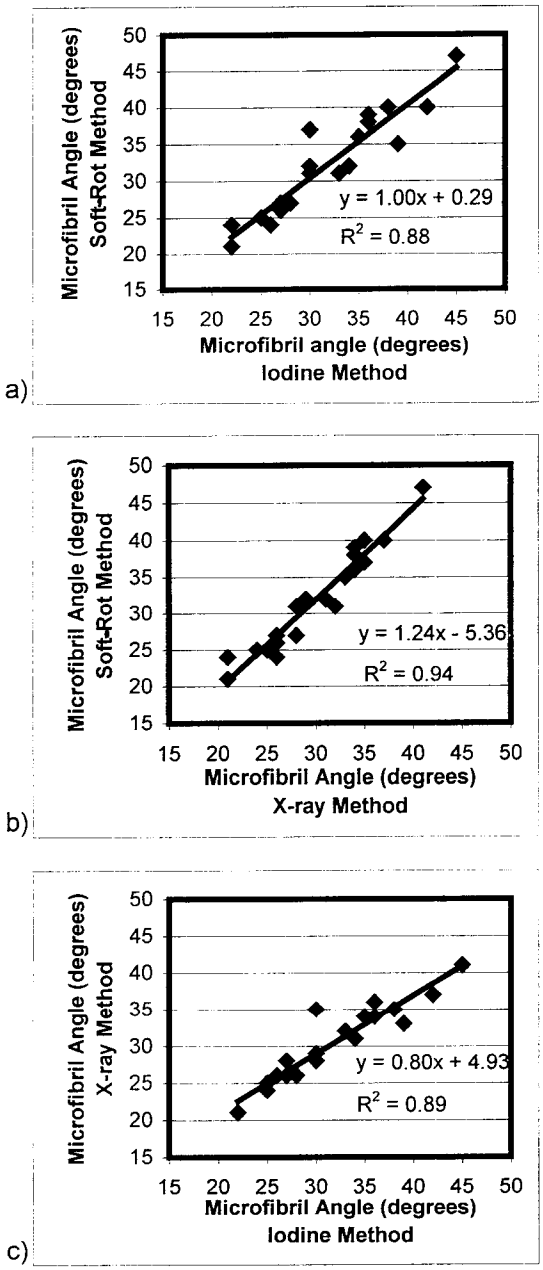

FIG. 4. Regression analyses comparing 3 methods of determining microfibril angle. Each point is the average of 100 cavities for each of the samples examined. A) Comparison of two direct methods, soft-rot and iodine (r^2 = 0.88). B) Comparison of a direct method, soft-rot, with an indirect method, X-ray (r^2 = 0.94). C) Comparison of the X-ray method to the iodine method (r^2 = 0.89).

TABLE 1. *Correlation coefficients (r) that compare the three methods of determining microfibril angle for latewood samples, earlywood samples, and all samples (earlywood and latewood combined). The results indicate that there is better agreement between methods with the latewood samples than with the earlywood samples. The greatest correlation was obtained when comparing the soft-rot and X-ray methods for earlywood and latewood combined.*

	Latewood	Earlywood	All
Soft rot–X-ray	0.93	0.92	0.97
Soft rot–Iodine	0.95	0.78	0.94
X-ray–Iodine	0.96	0.78	0.94

method, 95% confidence intervals for earlywood ranged from 0.8 to 2.8, while for latewood they ranged from 0.8 to 2.0. A similar outcome was observed with the iodine method. Earlywood variability ranged from 0.9 to 2.2, while for latewood the variability ranged from 0.8 to 1.4.

Correlations between methods were greater for latewood samples than for earlywood samples (Table 1). For the earlywood samples, the best correlation was obtained between the soft-rot and X-ray methods (0.92), the poorest when comparing the soft-rot to the iodine method (0.78) and the X-ray to the iodine method (0.78). When comparing the results for earlywood and latewood combined, better agreement was obtained between the soft-rot and X-ray methods (0.97) than between the iodine and X-ray methods (0.94).

There are several advantages of the soft-rot cavity method. First, the soft-rot cavities are relatively large structures that can be easily visualized in thin sections and individual fibers with either polarized light or Nomarski DIC microscopy. As the cavities are large and easily distinguished, one can focus through the cell wall without having concerns about the distortions caused by polarized light. Further, the cavities form primarily in the S2 layer and thus the fibril angle can be easily determined in both thin-walled earlywood cells and thick-walled latewood cells. This is an advantage over the reflected polarized light method (Page 1969) in which the effects of phase retardation give unacceptable results for thick latewood cells (El-Hosseiny and Page 1973). A possible limitation to this method is the time necessary for the fungi to attack the wood. Length of time to cavity formation could be minimized by careful attention to the moisture content of the sections during decay.

Iodine staining is not without criticism. The iodine staining occurs in all three cell-wall layers making it difficult to focus on the S2 layer when there is heavy staining in the S1 and S3, especially in thin-walled earlywood cells (Shupe et al. 1996). This could explain the relatively low r values obtained in this study for the earlywood samples when comparing the iodine method to either of the other methods (Table 1). It could also be a factor in the greater variability observed for earlywood samples. Iodine staining has also been criticized in that the pretreatments are harsh and cause extreme swelling and distortions of the cell wall. The soft-rot method, on the other hand, requires no harsh chemical treatments, swelling, or drying. The soft-rot cavity slides are permanent mounts, whereas in the iodine method, the staining is very transient, lasting only several hours.

The soft-rot method also demonstrates some advantages over the X-ray diffraction technique. The main advantage of X-ray diffraction is that it is a measure of the average microfibril angle of many tracheids. Thus, fewer samples need to be analyzed; however, it includes the ray cells and the other wall layers. Further, as the microfibril angle increases beyond 40 degrees, such as is common in juvenile wood, the determination of microfibril angle becomes increasingly difficult and variable. The soft-rot cavity method, on the other hand, only measures the angles in the S2 of tracheids and it is not limited by the microfibril angle. An angle of 55 degrees can be measured just as easily as an angle of 25 degrees. This ability to measure large microfibril angles is especially advantageous because of the increasing amounts of plantation-grown pine that has a large proportion of juvenile wood with its accompanying high microfibril angles.

CONCLUSIONS

We have demonstrated that the soft-rot cavity method is an accurate and viable method for the determination of microfibril angle. The soft-rot method agrees very well with both the iodine crystal method and the X-ray diffraction method. For the iodine crystal deposition method, earlywood samples were shown to have slightly lower correlations with the other methods than latewood samples, most likely caused by the inability to distinguish cell-wall layers. An advantage of the soft-rot method over the X-ray diffraction method is the ability to measure microfibril angles of any size including those of juvenile wood. An advantage over the iodine crystal method is the ability to distinguish the S2 in thin-walled cells.

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