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Within-a-Ring Variation of Lignin in Picea glehnii, by UV Microscopic Image Analysis

By

Tsutomu TAKANO**, Kazumi FUKAZAWA**
and Shigeo ISHIDA**

Introduction

Quantitative analysis of lignin using ultraviolet (UV) microscopy has been performed mainly by densitometrical analysis on UV micrographic negatives1,18,19. This indirect method is influenced by the development conditions of films and the unevenness of illumination. In order to avoid these influences we have developed UV microscopic image analysis5.

UV microscopic image analyser consists of UV microscope photometer and a microscopic image analysing system. The direct photometric scanning of UV microscopic image is carried out automatically, and the value of isodensity area of

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UV transmittance can be obtained. Since direct photometry is obtained through a small central diaphragm, there is no need to consider the uniformity of illumination. The merit of image analysis is believed that lignin distribution and wall areas are determined simultaneously within a growth ring directly and successively. Using this apparatus, FUKAZAWA and IMAGAWA divided the cell wall into the secondary wall and the middle lamella and obtained their areas and lignin contents. However, there were some problems in the reliability of these values. One of the purposes in the present paper is to discuss methodology of this system and to reexamine the procedures.

The study on lignin variation within a growth ring in detail has been reported chemically or with the aid of UV microscopy. In general lignin content of earlywood is higher than that of latewood and gradually decrease toward latewood. WU and WILSON examined minutely for lignin distribution of growth increments of five coniferous woods and mentioned that an anomaly was observed in the location of peak of lignin values which were frequently within, but not at beginning of the earlywood. FUKAZAWA and IMAGAWA reported that the intra-increment lignin pattern in juvenile wood was different from that in adult wood, examining in Abies sachalinensis. It was needed to explain the lignin variation pattern associated with adult and juvenile wood in a different coniferous wood. In this study, therefore, the variation of lignin within a growth ring and the relation between lignin contents and cell morphology both in adult and juvenile wood of Picea glehnii were investigated together with the examination of the methodology of UV microscopic image analysis.

**Materials and Methods**

Samples were obtained from a disk with 226 rings obtained at 9.2 m height above the ground of 367 year-old Picea glehnii tree with a diameter of 80 cm at breast height grown in the Teshio Experiment Forest, Hokkaido University. From the disk the 9th, 15th (juvenile wood), 107th (adult wood) and 217th (adult wood and sapwood) rings from the pith were selected for analysis. Small chips containing entire growth ring mentioned above were successively extracted with a mixture of alcohol-benzen (1:2) for 48 hours and aceton-water (1:1) for four days before embedding in epoxy resin. Three different sample rings aligned in parallel were embedded in one block. The thin transverse sections, with thickness of 0.5 μm, were cut with a diamond knife mounted on a LKB Type III ultramicrotome for UV microphotometry. Additionally Klasson lignin contents of wood meals prepared from the 14~24 th, 105~109 th and 215~219 th rings were determined. They were 27.5%, 26.4%, 25.6% respectively.

The apparatus was fully detailed in the previous paper. UV microscopic image analyser consists of UV microscope photometer (Carl Zeiss, Type MPM01) and microscopic image analyser (Rhesca, Type MPA730), and isodensity areas of UV transmittance were plotted and counted automatically. Direct photometric scanning at a wave length of 280 nm (band width of 5 nm) was carried out with
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A diaphragm diameter of 0.63 μm, 1 mm/min in scanning speed, and with scanning areas of 45~50 (transverse direction) × 50 (radial direction) μm. Isodensity images for each class of UV transmittance were plotted at a magnification of 800. The measurements were made with one section in the 9th ring, two sections in each of the 15th and 107th rings and four sections in the 217th ring.

Several UV transmittance areas were measured under 80%, 70% and in succession. The areas in each transmittance classes, such as 80~70% and 70~60%, were calculated from the measured values and their ratios to the entire cell wall area of transmittance under 80% were obtained. As shown in Table 1, the ratios of areas were multiplied by the relative absorbances based on absorbance values of transmittance in 75% respectively, and the sum of the product corresponds to the average of relative absorbance in this scanning area. Since lignin concentration is in proportion to absorbance according to the Lambert-Beer’s law, the relative absorbance corresponds to relative lignin concentration. The measurements were carried out from beginning to end of each growth ring in the same radial files successively.

Table 1. An example for measurement and calculation of lignin content

<table>
<thead>
<tr>
<th>Transmittance (%)</th>
<th>Relative absorbance (A)</th>
<th>Area (B)</th>
<th>A×B</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-70 (75)</td>
<td>1.00</td>
<td>0.210</td>
<td>0.210</td>
</tr>
<tr>
<td>70-60 (65)</td>
<td>1.50</td>
<td>0.314</td>
<td>0.471</td>
</tr>
<tr>
<td>60-50 (55)</td>
<td>2.08</td>
<td>0.312</td>
<td>0.649</td>
</tr>
<tr>
<td>50-40 (45)</td>
<td>2.78</td>
<td>0.095</td>
<td>0.264</td>
</tr>
<tr>
<td>40-30 (35)</td>
<td>3.65</td>
<td>0.057</td>
<td>0.208</td>
</tr>
<tr>
<td>30-20 (25)</td>
<td>4.82</td>
<td>0.013</td>
<td>0.063</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1.001</strong></td>
<td><strong>1.866</strong></td>
<td></td>
</tr>
</tbody>
</table>

The average relative lignin content of each growth ring in the section was calculated from all measurements of the ring. In order to calculate the absolute lignin contents from these average relative lignin contents and corresponding Klason lignin contents mentioned above, it is necessary to consider the ray tissue lignin. Percentages of ray tissue volume obtained in the same tree were 3.6% in the 9th and 15th rings and 5% in the 107th and 217th rings. The absorbance ratios of ray cell walls to tracheid secondary walls, measured within the same rings, were 2.41 in the 9th ring, 2.00 in the 107th ring and 2.12 in the 217th ring. It was assumed that the 9th and 15th rings were equal in absorbance ratio. Assuming that Klason lignin contents corresponded to relative lignin contents of ray tissue added to whole wall, the absolute lignin content could be calculated in each scanning area of each section; for example, in a section of the 107th ring, average relative lignin content of secondary wall was 1.76, that of total wall was 1.871, and absolute lignin content of 25.3% was obtained for relative lignin content of 1.871 as shown in following equation.
SCOTT and GORING reported that UV absorbance values were influenced by photolysis of lignin in UV light. In this investigation this phenomenon was also observed (Fig. 1). It took one minute and 22 seconds to expose a sample in scanning area to UV light for one series measurement. In Fig. 1, the reductions in absorbance were about two percent in secondary wall and one percent in cell corner middle lamella (ccML) for this time, and influence on UV absorbance values was as small as to be neglected.

![Graph](image)

**Fig. 1.** The decay of lignin UV absorbance for a 0.5 μm transverse section. S: Secondary wall. ccML: Cell corner middle lamella. At a wavelength of 280 nm (band width 5 nm).

**Results and Discussion**

1. **Validity of procedure**

Cumulative area distribution of cell wall is plotted against the relative lignin content from values in Table 1 (Fig. 2). The curve of cumulative areas distribution may obviously be divided into two parts, a straight line and a curve line. Lignin content of compound middle lamella (CML) is about twice as much that of secondary wall (S), and there is a large difference in lignin content between S and ML (broken line in the right part of Fig. 2). These two parts in the curve of cumulative area distribution, therefore, might correspond to S and ML respectively. ML part was separated from S part in

![Graph](image)

**Fig. 2.** Cumulative area curves against relative lignin concentration. Left part was drawn from data in Table 1. Right part was idealized curve. CC: Cell corner middle lamella.
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turning point of the cumulative area curve, and average values of relative lignin contents were determined by drawing a bisector in S and ML parts as shown in Fig. 2.

However, relative lignin contents, e.g. 1.866 in Table 1, are always larger than they calculated from these determined areas and lignin contents of S and ML; the former corresponds to the area in which oblique lines were drawn, and the latter corresponds to the area on the left of cumulative area curve (Fig. 2). In order to determine the areas and the lignin contents of S and ML, the relative lignin content values, i.e. the relative absorbance values, of plotted points were expediently modified as follows.

\[
1.00 (= \log 0.75/\log 0.75) \rightarrow 1.24 (= \log 0.7/\log 0.75) \\
1.50 (= \log 0.65/\log 0.75) \rightarrow 1.78 (= \log 0.6/\log 0.75) \\
\quad \text{etc.}
\]

For example, 1.886 was obtained as the relative lignin content from the curve of cumulative area distribution drawn from the values of modified relative lignin contents and the values in Table 1. Making a comparison between both of relative lignin contents in the same scanning area, e.g. 1.866 and 1.886, in 175 cases, the difference of 0.12 was obtained as the average value. It corresponds to about 0.1% in absolute lignin content, and differences are not more than ±1% in absolute lignin content.

In order to determine the co-ordinates of turning points, the two points which were first and second from turning point on the right were connected by a straight line, and the straight line on the left of turning point was drawn from the equation obtained by the least squares method.

For the purpose of examining the reliability of areas and relative lignin contents of S and ML obtained through this procedure, dot-counting method and point photometry of UV absorbance were carried out besides this. For dot-counting method, UV microphotographs which were at a magnification of 1900 and graph papers with 1 mm graduations were used. The percentage of S areas to the whole wall areas obtained by two methods, dot-counting method and image analysis, were compared statistically in the 13 same scanning areas. No significant difference was found between those two methods.

In the point photometry, UV absorbances of S and CML(t, r) were measured with a diaphragm diameter of 0.63 μm in eight scanning areas after image analysis. The average absorbances of S and CML(t, r) obtained by the point photometry were corrected by 1.02 and 1.01 respectively in order to make up for the reduction in UV absorbance by photolysis of lignin (see the foregoing paragraph). The relative lignin absorbances, based on the transmittance in 75%, were compared statistically between the values by the point photometry and the image analysis in the same scanning areas. In S, the difference of 0.11 as average value and the standard deviation of 0.136 were obtained. They correspond to about 1.54% and 1.90% in absolute lignin content respectively. In ML, the difference of 0.01 as average value and the standard deviation of 0.46 were obtained. They correspond
to about 0.14% and 6.4% in absolute lignin content respectively. No significant difference was found between them. It should be noted that ML lignin content obtained by the image analysis was the average values including CML(t, r) and cell corner ML(ccML) lignin. That obtained by the point photometry, however, was lignin content of CML(t, r) and did not include ccML lignin. If ccML lignin was appreciated, lignin content of ML by the point photometry should increase. More examination will be need to check the procedure concerning ccML lignin.

Since no significant difference was found, it would be possible to examine the variation of lignin content within a growth ring in spite of this unsolved problem.

2. Variations of secondary wall area and lignin percentage

The variations of S and ML area percentages of scanning areas, the S area percentage of total wall and S lignin percentage of total wall were shown in Fig. 3; in examined the four rings, their variation patterns were almost similar, and those in the 15th and 107th rings were shown. Although S lignin content is lower than ML lignin content, S lignin mostly occupies the total wall lignin owing to the larger area of S. The S lignin percentage of total wall, therefore, is almost influenced by the S area percentage, not by the S lignin content.

The variation of S lignin percentage was similar to that of S area percentage. S area percentage of total wall was low in the initial zone of earlywood, and highest in the terminal zone of earlywood, and decreased toward the ring boundary. These variations are explained by the variations of S and ML areas of scanning area. S area was smallest in the initial zone of earlywood, and largest at the terminal latewood. ML area was also largest at the terminal latewood. S area began to increase in the late earlywood owing to the decrease in radial diameter of tracheids (Fig. 6), while ML area began to increase in latewood later than S area. This is the reason,
why S area percentage of total wall was highest in the late earlywood. In latewood
S area increased owing to the thickening of S and the reduction in radial diameter
of tracheids. The increasing rate of ML area, however, was higher than that of
S area, therefore, S area percentage of total wall decreased in latewood.

The S area and lignin percentages of total wall in latewood were obviously
larger than in earlywood in former investigations\(^5,11,12,14,21,25\). FUKAZAWA and IMAGAWA\(^8\), however, reported the larger S area and lignin percentages in earlywood
and latewood in *Abies sachalinensis*. In the present paper the difference of S area
and lignin percentages between earlywood and latewood were small in adult wood,
while the S area and lignin percentages in earlywood were larger than in latewood
in the 9th and 15th rings (Table 2). Since the differences of S area and lignin
percentages between both woods varied with species on the literature\(^5,11,12,14,21,25\)
these would partly result from the characteristics of species or the variations between
individuals. If S areas were measured in a some typical part of earlywood or late­
wood and averaged, they should not show the true average values. It was noted
that the positions of maximum or minimum S area percentages were in earlywood
within a growth ring.

<table>
<thead>
<tr>
<th>Ring No.</th>
<th>Lignin content (%)</th>
<th>S % of total wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wall</td>
<td>S</td>
</tr>
<tr>
<td>217</td>
<td>EW</td>
<td>24.9</td>
</tr>
<tr>
<td></td>
<td>LW</td>
<td>23.9</td>
</tr>
<tr>
<td>107</td>
<td>EW</td>
<td>25.5</td>
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<tr>
<td></td>
<td>LW</td>
<td>25.2</td>
</tr>
<tr>
<td>15</td>
<td>EW</td>
<td>25.6</td>
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<tr>
<td></td>
<td>LW</td>
<td>26.5</td>
</tr>
<tr>
<td>9</td>
<td>EW</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>LW</td>
<td>27.9</td>
</tr>
</tbody>
</table>

3. Lignin variation within a growth ring

The variations of lignin contents within growth rings were shown in Fig. 4.
In the figure, “Wall” is the average lignin content value of cell wall, and “S” and
“ML” are lignin contents of S and ML. Since ccML could not be separated from
CML\((t, r)\) in this investigation, “ML” means the average lignin content of CML\((t, r)\)
and ccML here. The boundary between earlywood and latewood was set at 50% of
whole wall area on scanning area according to the literature\(^9\). Results in the
107th and 217th rings were similar to the previous investigations\(^5,25\). Lignin content
was highest at the initial zone of earlywood, the first or second cell, decreased to
the minimum value, and increased toward the ring boundary. The position where
lignin content was lowest was around the boundary between earlywood and late­
wood.
A somewhat different variation pattern was found in the 9th and 15th rings. Lignin content varied wavelingly and was also highest at the initial zone of earlywood. The position of minimum lignin content within a growth ring was variable, and there was no significant difference among the lignin contents at the three bottoms of wavy line. The positions of top and bottom in the wavy line within growth ring were similar in the 9th and 15th rings.

The variation patterns of S lignin content always paralleled that of average cell wall lignin content. This shows that the latter depends on the former. ML lignin contents varied larger than S, and its variations were irregular except for increasing within latewood. All this increasing, however, would not be the actual lignin variation. It seemed that the CML(t, r) widths were smaller than the diameter of diaphragm, 0.63 μm, in the most parts of growth rings. It was reported that CML(t, r) width was 0.45 μm in black spruce (earlywood)⁹ and that the thickness of ML fragment from fibers of mechanical pulp of spruce was 0.39 μm⁹. Lignin content of ML obtained was expected to be smaller than the true lignin content of CML(t, r). Moreover the increase in CML(t, r) width and in ccML areas caused the increase
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in ML lignin content within latewood. The CML(t, r) width increase in latewood, especially in the terminal zone of radial wall was shown in Photo 1, 2 and 6. Additionally the proportion of ccML area to CML(t, r) area increased in latewood accompanied with the reduction in radial diameter of tracheids.

Surveying the literature on CML(t, r) lignin contents of earlywood and latewood, they were 49.7% and 60% in black spruce, 56% and 60% in Douglas-fir and 49% and 51% in loblolly pine. The lignin content of ML fragments in mechanical pulp of spruce determined by UV microscopy was 57.1%. WHITING and GORING separated the tissue fractions of ML and found that their lignin content was 60% by chemical method. In this investigation ML lignin contents had the range of 47.8-53.2%. These were a little lower than the values on the literature. This would be attributed to the smaller diameter of diaphragm than CML(t, r) width.

The large variation of ML lignin content was caused by the variation of ccML area, the variation of ccML lignin content and the existence of bordered pits in the scanning areas. It was observed that size and lignin content of ccML were variable (Photo 3). When a scanning area contained a bordered pit cut as in Photo 3, the area of high lignin content increased, and as a result it was regarded as ML following the procedure in this study. Lignin content of ML, however, did not increase always, therefore, its influence on lignin content was small.

Absorbances of lignin were determined in detail across the ring boundary (Fig. 5). Besides the data obtained by the image analysis, lignin absorbance of ccML was determined by the point photometry. Lignin absorbances of S and ML varied successively across the ring boundary, except that the discontinuity of absorbance in UV microphotographs was frequently observed at the ccML of ring boundary (Photo 1, 4 and 6). Lignin contents of ccML varied largely in the terminal zone of latewood, and a drop of ccML lignin content in there reported by SAKA et al. was not observed.

The average values in earlywood and latewood were shown in Table 2. Lignin content of earlywood is generally higher than that of latewood. The difference of lignin contents between earlywood and latewood in spruce was smaller than in other species, and in some cases latewood lignin contents were higher. It was possible that samples contained the compression wood in these cases. In this study differences of lignin contents between maximum and minimum were 6.82 in the 9th ring, 5.81 in the 15th ring, 5.29 in the 107th ring and 4.54 in the 217th ring. These values were larger than the values in spruce wood on the literature. In the 9th and 15th rings lignin content of latewood
was higher than that of earlywood. It should be noted, however, that the positions where lignin content was highest or lowest were within earlywood as seen in Fig. 4.

The higher lignin content of S3 layers was reported\(^8,14,19\). In this study S3 layers had apparent higher UV absorbance than S2 layers in latewood of juvenile wood as reported by FUKAZAWA and IMAGAWA\(^8\) (Photo 2). In latewood of adult wood the higher UV absorptive S3 layers was also observed in the cell corner (Photo 1), but not always. It was not observed in earlywood of adult or juvenile wood (Photo 3 and 5).

4. Relation between lignin contents and cell morphology

Using UV microphotographs, radial diameter of tracheids and lumina were measured to examine the relation between lignin contents and cell morphology. Variations radial diameter of tracheids and cell wall thickness were shown in Fig. 6.

![Fig. 6. Variations of radial cell wall thickness and radial diameter of tracheids within growth rings in the 15th and 107th rings.](image)

Radial diameter of tracheid began to decrease in the latter half of earlywood and was smallest at the terminal zone of latewood. Lignin content of S and cell wall thickness appeared to have an opposite relation in the 107th and 217th rings. Comparing these in detail, however, it could be found that the position of maximum cell wall thickness did not agree with that of minimum lignin content. Examining the variations in the 9th and 15th rings, apparent relation of both was not observed. In these rings the position of minimum lignin content was apparently away from that of the thickest cell wall. The variations corresponded to the wavy lines of lignin contents were not observed in the variation lines of radial diameter of tracheids or cell wall thicknesses. Both variations partly agreed with lignin variation in all growth rings examined.

5. Juvenile and adult wood

Chemical composition of wood varies in stem cross sections. Lignin decreases from pith to bark\(^\text{6,7}\).
Larson studied chemical composition in red pine, dividing into earlywood and latewood. In juvenile wood lignin variation was more or less larger than in adult wood, and lignin content of earlywood was always higher than that of latewood. Sastry and Wellwood determined some cellular characteristics of earlywood and latewood in Douglas-fir of 500 year-old. Holocellulose, alphacellulose, and crystallinity index were higher in latewood than in earlywood of the 10th ring which was the nearest ring to the pith of all examined rings. Lignin content had to be higher in earlywood than in latewood.

Fukazawa and Imagawa reported the peculiar lignin variation pattern of juvenile wood in Abies sachalinensis; lignin content increased from earlywood to latewood. In this study, as shown in Fig. 4, variation pattern of lignin in juvenile wood was also different from that in adult wood.

Sarkanen and Hårgert and Wu and Wilson noted the irregularity of lignin content in latewood. Fukazawa and Imagawa reported that lignin contents had the ranges of 27.2–28.1 in earlywood and 23.5–29.9 in latewood. In this study lignin contents had the ranges of 24.4–25.6 in earlywood and 23.9–27.9 in latewood. Range in latewood was larger than that in earlywood. If lignin content of earlywood was roughly constant and that of latewood was variable, variation pattern of lignin content in the 9th and 15th rings could be expected to be medium pattern between variation pattern of juvenile wood reported by Fukazawa and Imagawa and that of adult wood. Varying as reported by them in juvenile wood, however, lignin content would increase from pith to bark, and this contradicts the general tendency, decreasing from pith to bark. They suggested that variation pattern of lignin reported by them in juvenile wood might be attributed to the characteristics of Abies species. Lignin content would vary around the constant level in juvenile wood, and lignin content of latewood would decrease toward adult wood, and general variation pattern within growth ring of adult wood might be formed finally.

This change was similar to the variation of cell wall thickness from pith to bark. Cell wall thickness of tracheids in latewood increases progressively from pith to bark, while that in earlywood is increases from pith to bark and the increasing ratio of that in earlywood is smaller than that in latewood. The degree of variation varies with species. Wu and Wilson showed that the range of lignin content within growth ring depended on nature of the earlywood-latewood transition. The earlywood-latewood transition is more or less abrupt in red pine and abrupt in Douglas-fir. The differences of lignin contents between earlywood and latewood are great in both species. In these species in which the earlywood-latewood transition is abrupt, variation pattern of lignin content might not change or be difficult to change in juvenile wood.

Conclusions

The variation of lignin content within a growth ring was investigated by UV microscopic image analysis.
On the methodology, we examined the validity of procedure and ascertained to be reliable values of lignin contents and areas of secondary wall (S) and middle lamella (ML). Following the procedure described here, it would be possible to examine the lignin variation within a growth ring.

Variation pattern of lignin content from earlywood to latewood was similar to that reported previously in adult wood\(^{2,3}\), and it was something different from that in juvenile wood. Lignin variation pattern would be changed by the cambial age.

The relation between this variation and cell morphology was not clearly obtained.

It was useful to determine the lignin contents of S and ML or the areas of them successively within a growth ring using the image analyser. The accurate information about their variations within a growth ring should not be obtained from the average or partial values in earlywood and latewood. For example, lignin content of latewood in the average value was higher than that of earlywood in juvenile wood, though the positions of maximum and minimum values were within earlywood.

In order to make use of the characteristics of image analyser, the automatism of measurements will be advanced further. More accurate information will be obtained by examining more samples.

References


2. 年輪内リグニン分布の変化は従来の報告と同様であった。すなわち早材イニシャル部分でリグニン濃度は最大であり、その後漸減して早材境界付近で最小となる。そして年輪ターミナル部分に向かって増加する。しかし、成熟材部では異なるパターンが見られた。最大値の場所は同じであるが、その後周期的に変動し明らかな最小値部分を示さなかった。これらの変動はリグニン濃度の変動に平行しており、一方MLリグニンは一定の変動パターンを示さなかった。

これらの値を早材材別に平均したところ、未成熟材部では晚材リグニン濃度が早材よりも高かった。しかし、この最大値と最小値はいずれも早材部内にあった。

3. 年輪内における仮道管の半径方向径および壁厚の変動を調べ、リグニン濃度変動と比較したが、明らかな関連性は認められなかった。

4. 成熟材部と未成熟材部のリグニン変動パターンの違いから、パターンは樹幹内で変化するものと考えられる。

以上の結果から測定装置および測定、算出法の有効性が確認された。画像解析装置の使用により、細胞壁におけるリグニン濃度別の面積割合の連続的な測定が可能となった。

今後はより詳細に、年輪内リグニン変動パターンを樹幹内、そして異なる樹種間で比較していくことが望まれる。

**Explanation of photographs**

**Note:** These are UV microphotographs of 0.5 µm thickness transverse sections taken at a wavelength of 280 nm.

**Photo 1.** Ring boundary between the 217th and 218th rings from pith. Width of middle lamella in radial wall of latewood is large. S3 layer of higher UV absorbance is observed at the cell corner of latewood.

**Photo 2.** Latewood in the 8th ring. S3 layers of higher UV absorbance are obvious.

**Photo 3.** Earlywood in the 217th ring, in the middle of growth ring. A section of bordered pit is observed. In larger cell corner middle lamella, there is a part of lower UV absorbance.

**Photo 4.** Ring boundary between the 216th and 217th rings. Discontinuity of absorbance is observed at the cell corner middle lamella of ring boundary.

**Photo 5.** Initial earlywood in the 9th ring.

**Photo 6.** Latewood in the 217th ring.