

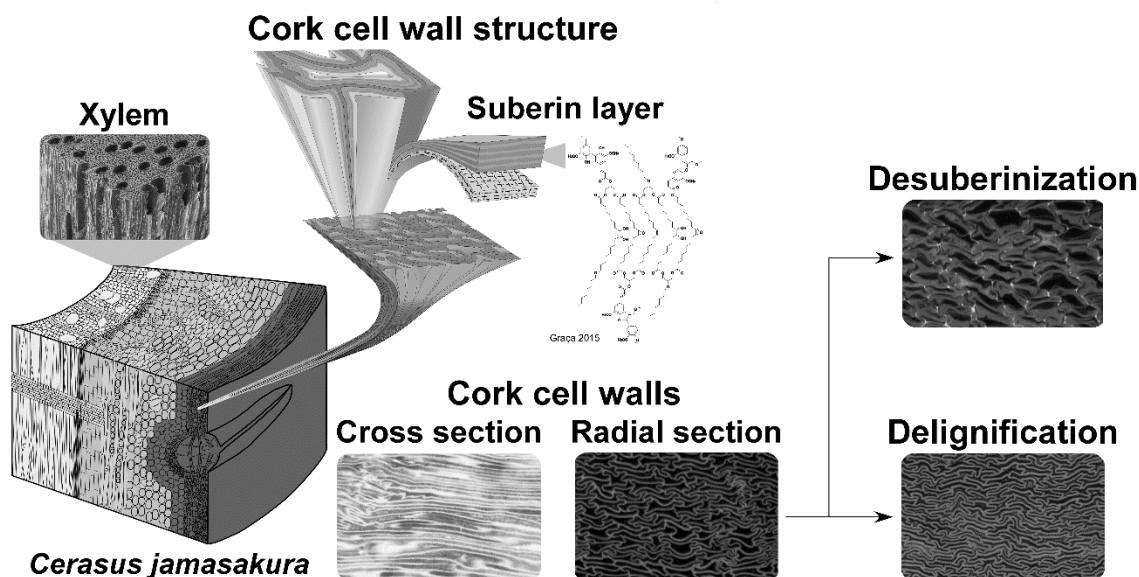
Effect of Desuberinization and Delignification on the Cork Cell Walls of *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba using FTIR Spectroscopy and Microscopic Observation

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DOI: 10.15376/biores.19.3.5129-5140

GRAPHICAL ABSTRACT



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Hayato Saito,^a Takahisa Nakai,^{a,*} Keisuke Toba,^{a,b,*} and Toru Kanbayashi^b

Bark, the outermost tissue, plays an important role in protecting trees from damage induced by living organisms and the surrounding environment. Bark differs from the xylem primarily by the presence of suberin in cork cell walls. However, few studies have examined the role of suberin and its interactions with other chemical components in the cork. Consequently, this study aimed to understand the distribution of chemical components, including suberin and lignin, and their respective roles in cork cell walls, using *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba. Suberin and lignin were gradually and selectively removed from thin strip specimens. Fourier transform infrared (FTIR) spectroscopy suggested that desuberinization removed both suberin and part of the other matrix substances within a few minutes of treatment, whereas delignification exclusively removed lignin. Further microscopic observation revealed that suberin present was mainly in the secondary wall of cork cells, whereas lignin was present in both the tertiary wall and compound middle lamella. In addition, the cell wall collapse of the cork was only found in desuberinized specimens, whereas delignified specimens only showed monotonic contraction. Taken together, these results suggest that the presence of suberin in the cork contributes to the shape stability of cork cell walls.

DOI: 10.15376/biores.19.3.5129-5140

Keywords: Bark; Phellem; Suberin; Lignin; Distribution; Collapse

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INTRODUCTION

Bark affects the appearance of trees and usually occupies 9% to 15% of the stem (Feng *et al.* 2013). It consists of cellular tissues outside the vascular cambium and covers the outermost stem surface. For example, the bark of *Quercus suber*, well known as a raw material for cork, is used for many applications, including as a bottle stopper and in thermal insulation panels (Leite and Pereira 2017). However, the uses of bark remain limited, even regarding traditional craft production. Due to its lower commercial importance, scientific analysis of the material properties of bark remains less developed.

One remarkable aspect of bark is the diversity of its tissue structure. In general, the bark formed on the outside of the xylem can be divided into inner and outer bark (Fig. 1a). The former consists of a secondary phloem, whereas the latter corresponds to the innermost periderm as well as subsequent periderms with the tissue isolated by them (Angyalossy *et al.* 2016).

The periderm, the secondary tissue produced by trees, consists of phellogen, phelloderm, and cork (hereinafter referred to as phellem) (Shtein *et al.* 2023). The most of periderm is formed by differentiated dead cells, with some exception such as phellogen and phelloderm (Angyalossy *et al.* 2016). Of these tissue types, the phellem consists of three layers with lignified and suberized cell walls (Graça 2015), and the ratio of the chemical components present can vary greatly depending on the tree species (Feng *et al.* 2013).

The main difference between phellem and xylem cell walls is the presence of large amounts of suberin in phellem, which is one of its characteristic components. Suberin is an aliphatic and aromatic cross-linked polymer with hydrophobic properties that plays a key role in protecting living trees from surrounding organisms and environmental change (Pereira 2015). However, the spatial configuration of the bark cell walls and their relationship between spatial organization and the presence of suberin and lignin, among other components, remains unclear (Gandini *et al.* 2006).

Previous studies have investigated the structure of phellem cells and their chemical components. For example, Shibui and Sano (2018) demonstrated the anatomical structure and formation process of the periderm of *Betula maximowicziana* using a series of microscopic observations. Other researchers have also investigated the fundamental chemical components of phellem, including its molecular structure and biosynthesis using *Quercus suber* (Graça 2015; Serra and Geldner 2022). In addition, several specific mechanical properties of the phellem have been determined using *Cerasus* and *Betula* tree species. These include the influence of the moisture condition, specific microscopic structural differences from the xylem, and selective damage to chemical components (Kobayashi *et al.* 2019; Saito *et al.* 2020; Kiyoto and Sugiyama 2022). Furthermore, the role of chemical components in the phellem cell wall was investigated through microscopic observations after removal of suberin and lignin. For example, Pereira and Marques (1988) demonstrated that the removal of suberin involved substantial voids throughout the cell walls, whereas removal of lignin caused the cell wall collapse in *Q. suber*. Moreover, Kiyoto and Sugiyama (2022) demonstrated that the inner layers of early phellem, consisting of thin walls, lost their shape, whereas those of late phellem, consisting of thick walls, remained in shape after removal of suberin treatment in *Betula platyphylla*.

Despite the above intense research progress, the roles that specific chemical components play in the phellem remain poorly understood, as does their distribution in phellem cell walls and which interactions they have with other components. The main focus of this study was to investigate the characteristics of the phellem of *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba., which has been traditionally used such as the fastener of lunch boxes of Japanese craft products. The shape of the phellem cells in this tree species is known to have a distinctive shape compared to other species: cells are compressed in the radial direction (Saito *et al.* 2021). It is considered that some kind of phenomena must be developed in phellem cell walls to maintain the unique cell shapes, such as the presence of chemical components or stress conditions, but the details are still unclear. Thus, the purpose of this study was to determine the distribution and function of key chemical components of the phellem, including suberin and lignin. Based on the gradual removal of each chemical component, the effects of chemical distribution using *C. jamasakura*.

EXPERIMENTAL

Preparation of Bark Specimens

Bark specimens used were taken from the branches of *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba. Samples were collected on October 25, 2019, at Mie University Experimental Forest, Mie Prefecture, Japan (34°27'29.2" N, 136°13'52.0" E). The diameters of sampled branches ranged from 12 to 14 cm. After the branches were removed using a handsaw, they were preserved in a freezer (JF-NC205F, Haier, Osaka, Japan) at $-20\text{ }^{\circ}\text{C}$.

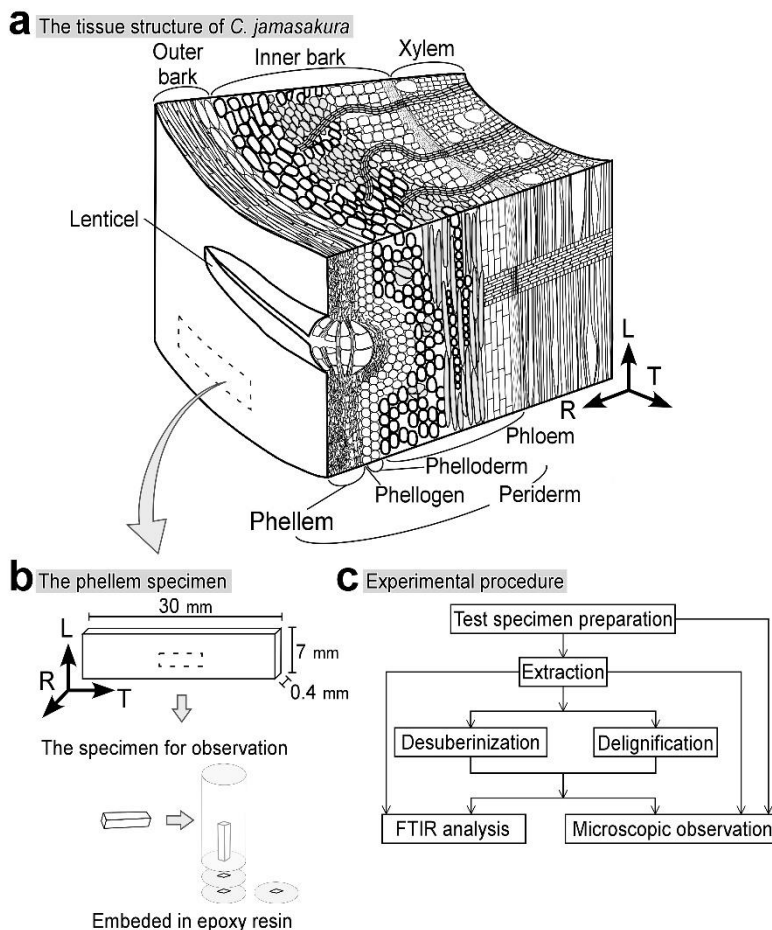


Fig. 1. Anatomical structure of bark tissue of *C. jamasakura* and specimen preparation: a: Schematic diagram, b: strip specimen of phellem, and c: experimental procedure

Desuberinization and Delignification

Phellem specimens were cut from sampled branches, as shown in Fig. 1b. Each specimen was adjusted to $7 \times 0.4 \times 30\text{ mm}^3$ (longitudinal \times radial \times tangential) using a utility knife. Next, specific chemical components were gradually and selectively removed by the following procedures (Fig. 1c).

Extractives were removed as a pretreatment using a Soxhlet extractor with dichloromethane, ethanol, and hot water (Pereira 1988). Briefly, specimens were refluxed until a clear solution was obtained in the upper part of the Soxhlet column. The authors used three extraction times: 16, 20, and 48 h. After these treatments, some specimens were

air-dried in an experimental laboratory, while other specimens were dried in an electric oven (DK400T, Yamato Scientific, Tokyo, Japan) at 105 °C for 24 h. Specimens were then weighed to calculate the ratio of chemical components present.

Next, half of the extracted specimens were subjected to desuberinization based on a depolymerization reaction (*i.e.*, methanolysis) using a methanolic solution of 3% (v/v) NaOCH₃ (Pereira 1988). After soaking the specimens in this solution for 6 h, they were refluxed and collected at 1 min, 10 min, 30 min, 1.5 h, and 3 h time points. The yields of these time points were 9.3%, 10.8%, 13.8%, 19.1%, and 32.7%, respectively.

The other specimens were subjected to delignification using an aqueous solution of 4% (w/v) NaClO₂ (Klaudiz 1957). After soaking the specimens in the solution for 6 h, they were refluxed and collected after 30 min, 6 h, 24 h, 48 h, and 96 h. The lignin yields at these time points were 3.6%, 9.4%, 11.7%, 24.4%, and 31.4%, respectively.

Microscopic Observations

Subsequently, both untreated phellem specimens and specimens subjected to the removal treatments described above were gradually dehydrated in a graded ethanol series (50, 70, 85, 95, 100, 100, and 100 % (v/v) ethanol for each 45 min). Ethanol was removed *via* a graded acetone/ethanol series for 1.5 h, and samples were then soaked in acetone for 3 h. Next, acetone was substituted with epoxy resin *via* a graded epoxy resin/acetone series for 24 h and the samples were finally soaked in an epoxy resin for 48 h. This epoxy resin consisted of Epon 812, dodecenyl succinic anhydride, methyl nadic anhydride, and 2,4,6-tris (dimethylaminomethyl) phenol. These reagents were purchased from Taab Laboratories Equipment (Aldermaston, Berkshire, UK). The volume fractions used were 100:1, 25:1, 75:1, and 1:1. After fixing, 1- μ m-thick radial sections were prepared using a rotary microtome (HistoCore MULTICUT R, Leica, Wetzlar, Germany). Some of these sections were then stained with 0.5% (w/v) safranin aqueous solution, while others were stained with 1% (w/v) phloroglucinol in ethanol, followed by treatment with phloroglucinol-HCl. Because this color reaction is transient, observations were performed within 15 min after staining. Microscopic observations were performed using a light microscope (E600W, Nikon, Tokyo, Japan) equipped with a digital camera (DS-Fi2, Nikon, Tokyo, Japan). Fluorescent images of stained and unstained tissues were also captured using a fluorescence microscope (BX41 OLYMPUS, Tokyo, Japan) equipped with a digital camera (DP73, OLYMPUS, Tokyo, Japan), an LED light source (X-Cite XLED1, OLYMPUS, Tokyo, Japan) with 330 to 385 nm excitation filter, a 400 nm dichroic mirror, and a 420 nm emission filter.

Fourier Transform Infrared Spectroscopic Analysis

Fourier transform infrared (FTIR) spectra were obtained using an FTIR spectrophotometer (FT/IR4700, Jasco, Tokyo, Japan) equipped with an attenuated total reflectance (ATR) device and a single-reflection diamond crystal (ATR Pro One View, Jasco, Tokyo, Japan). The FTIR/ATR analyses were then conducted directly on the surface of specimens following treatment. The incident angle of the infrared light through the diamond crystal was 45°. The spectral resolution, accumulation number, and measurement range were 4 cm⁻¹, 16 times, and 4,000 to 400 cm⁻¹, respectively. The absorbance ratios were calculated based on the respective peaks of FTIR spectra appearing at 2917, 2849, and 1515 cm⁻¹ to the peak at 1104 cm⁻¹ deriving from the stretching of glucose ring. (Pandey and Pitman 2003).

RESULTS AND DISCUSSION

Distribution of Chemical Components in Phellem Cells

Figure 2 shows the radial sections of bark tissue and part of the xylem. It is clear that untreated xylem and bark tissues showed a color reaction when visualized using phloroglucinol-HCl staining. In particular, the area of the phellem showed a stronger reaction with dark color (Fig. 2a). This color indicates the presence of lignin (Donaldson and Williams 2018; Kitin *et al.* 2020). In contrast, fluorescence microscopy revealed that significant autofluorescence was only found in the phellem (Fig. 2b). Biggs (1985) and Wilms *et al.* (1990) reported that staining with phloroglucinol-HCl can quench the autofluorescence of lignin. The fact that fluorescence was observed only in the phellem suggests that the fluorescence was caused by substances other than lignin contained in the phellem. Further observations were also performed on the phellem itself (Fig. 3). Most of the color produced during phloroglucinol-HCl staining was found in the compound middle lamella (CML), whereas such staining was rarely found in the secondary (Sec) and tertiary (Ter) walls (Fig. 3a). In addition, fluorescence microscopic observation showed that bright autofluorescence occurred only in areas containing secondary cells (Fig. 3b). Moreover, due to safranin staining, the tertiary walls were stained but the secondary walls were not (Fig. 3c). Therefore, the secondary walls of the phellem are rarely lignified, even though the other cell walls are lignified. Given the chemical components of the phellem, it was hypothesized that the autofluorescence found in the secondary walls is due to the presence of suberin (Biggs 1985). This chemical component, which is distributed only in the secondary walls, is one of the main components of the phellem and consists of both aromatic and aliphatic compounds.

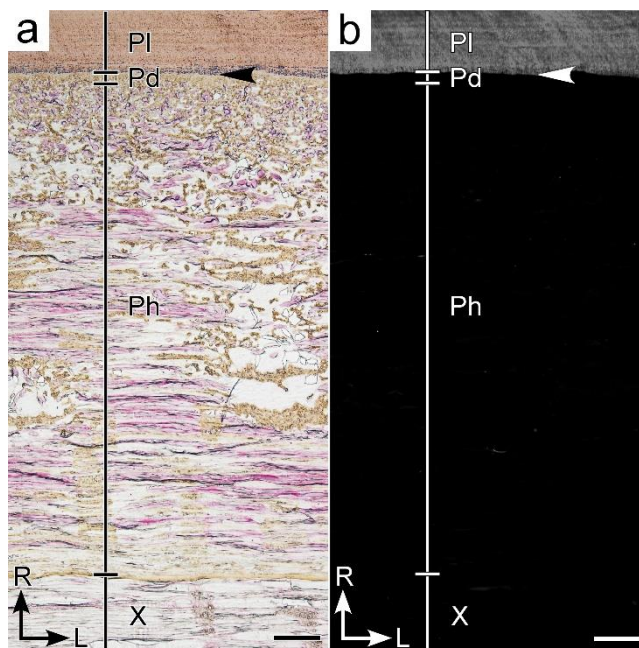


Fig. 2. Radial section of bark. a: Optical microscope image of the tissue near the cambium; b: fluorescence microscope image of the area shown in a. Bark tissues in a and b were stained with phloroglucinol-HCl. Bars in Figs a and b indicate 200 μ m. Abbreviations are as follows: Pl: phellem. Arrowheads: phellogen. Pd: phelloderm. Ph: phloem. X: xylem.

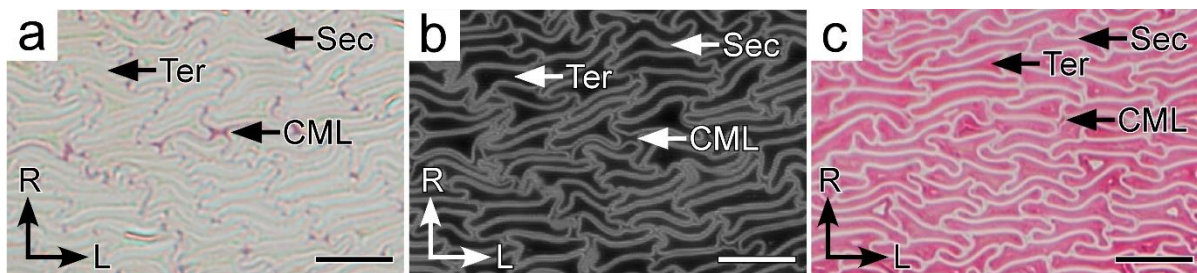


Fig. 3. Radial section of phellem cells. a and c: optical microscope images of the phellem; and b: Fluorescence microscope image of the area shown in a. phellem cells in a and b were stained with phloroglucinol-HCl, and the image shown in c image was stained with Safranin. Bars in Figs a, b and c indicate 10 μ m. Abbreviations are as follows: CML: compound middle lamella (including primary wall). Sec: secondary wall. Ter: tertiary wall.

Microscopic Observations of the Removal of Chemical Components

Figure 4 shows the changes in autofluorescence in the phellem during the desuberinization process. It was found that autofluorescence due to the presence of lignin was maintained in unstained phellem specimens even after 1.5 h of treatment (Fig. 4UC). In contrast, this autofluorescence disappeared in phloroglucinol-HCl-stained specimens. As mentioned earlier, phloroglucinol-HCl staining can quench lignin fluorescence. Thus, the bright areas in Fig. 4UB and UC indicate areas in which lignin remained. The disappearance of fluorescence in Fig. 4SB and SC indicates that most of the suberin was removed even after 30 min of desuberinization. It should also be noted that most secondary cell walls lost their cell shape in the radial direction during treatment (Fig. 4UB and UC). These changes in phellem cell shape confirm the results of a previous study (Pereira and Marques 1988). As previously noted, phellem of *B. platyphylla* characterizes an early phellem-late phellem arrangement (Kiyoto and Sugiyama 2022). However, this structural characteristic was not clearly observed in phellem of *C. jamasakura*. Consequently, they were uniformly desuberinized after 1.5 h of treatment.

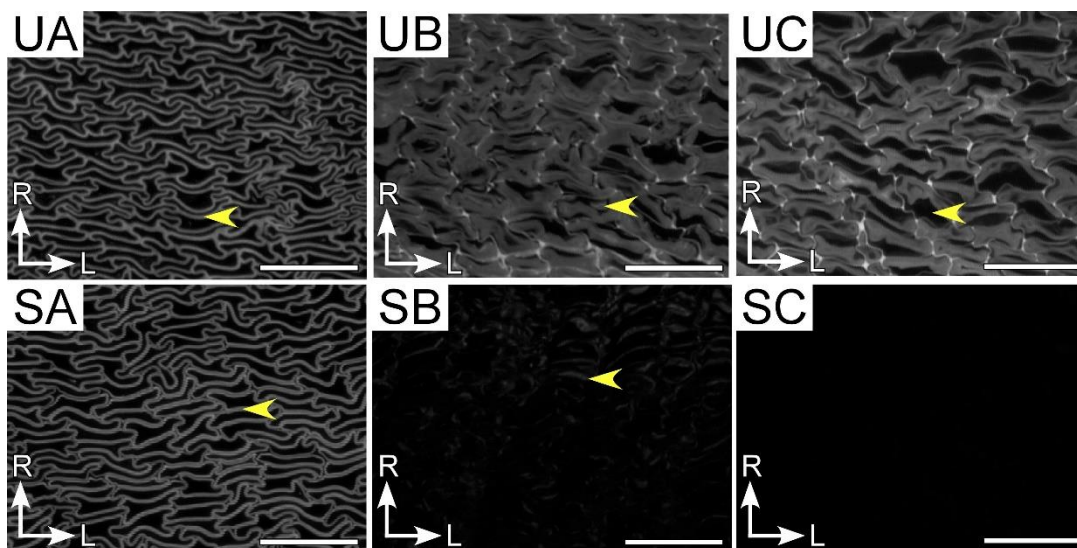


Fig. 4. The fluorescence micrographs of the radial section of phellem treated with desuberinization for 0 min (UA, SA), 30 min (UB, SB), and 1.5 h (UC, SC). U: Unstained; S: Stained with phloroglucinol-HCl. Bars in all figures indicate 10 μ m. Yellow arrowheads: Secondary wall (cannot be observed in SC)

Figure 5 shows changes in phellem autofluorescence during the delignification process. Overall, most phellem cells exhibited autofluorescence, regardless of whether they were stained with phloroglucinol-HCl or the duration of delignification. Furthermore, it was noted that the folded tertiary walls of the phellem cell loosened after 24 h of delignification (Fig. 5UB and SB), after which they became thinner and aggregated (Fig. 5UC and SC). Thus, the differences in cell shape following desuberinization treatments suggest that suberin may maintain cell shape within the phellem.

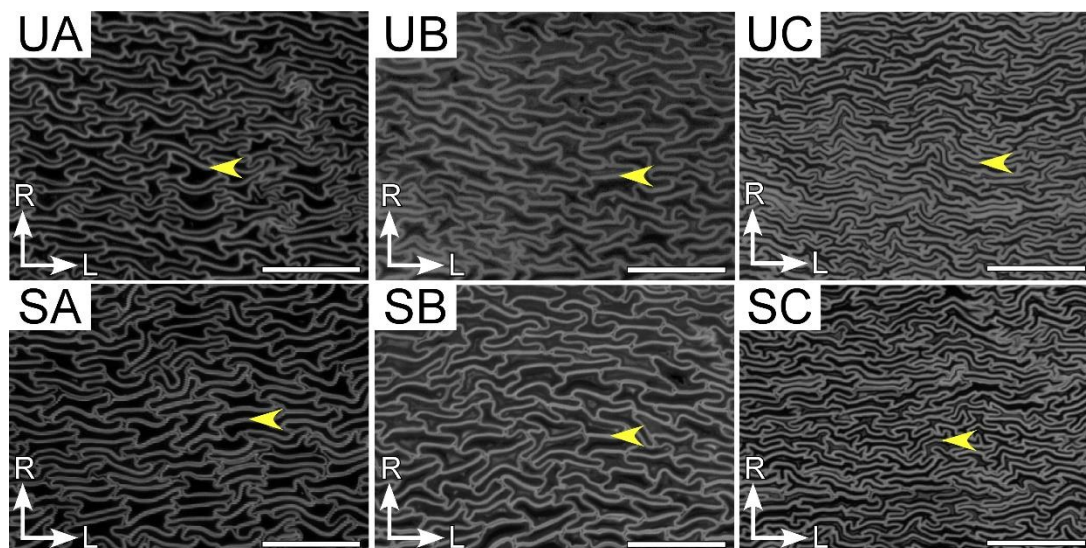


Fig. 5. The fluorescence micrographs of the radial section of phellem treated with delignification for 0 min (a-u, a-s), 24 h (b-u, b-s), and 96 h (c-u, c-s). u: Unstained; s: Stained with phloroglucinol-HCl. Bars in all figures indicate 10 μm . Yellow arrowheads: Secondary wall

FTIR Analysis of the Removal Process of the Chemical Components

Figure 6 shows typical FTIR spectra before and after desuberinization, including measurements at the 1 min, 10 min, 30 min, 1.5 h, and 3-h time points. The absorbances at 2917 and 2849 cm^{-1} are mainly derived from C–H bonds in the aliphatic chains of suberin (Lopes *et al.* 2000); these absorbances showed the significant reduction, especially in the early stages of the treatment. The absorbance at 1158 cm^{-1} , partially derived from the O–C=O structure found in the aliphatic esters of suberin (Şen *et al.* 2012), showed a reduction that was correlated with the above absorbances. In addition, the absorbance at 1736 cm^{-1} , likely derived from the C=O bond of suberin ester groups (Lopes *et al.* 2000), almost disappeared after only 1 min of desuberinization. The FTIR spectra of the extracted suberin obtained from the phellem showed significant peaks at the wavenumbers described above (Fig. 7), and it should be noted that these absorbances decreased or disappeared after 3 hours of treatment. Therefore, most of the suberin in the phellem cell walls was likely removed by 3 h of treatment. The authors also found other absorbance reductions following desuberinization, including the wavenumber at 1515 cm^{-1} , which is likely associated with the C=C of lignin aromatics (Lopes *et al.* 2000; shown in Fig. 6). Over the course of the treatment, it was also observed differences in absorbance in the spectrum from 1500 to 1200 cm^{-1} , which is linked to matrix substances such as hemicellulose and lignin. This indicated that desuberinization, *i.e.*, a chemical treatment with sodium methoxide, may damage not only suberin but also hemicellulose and lignin. However, absorbances in the range from 1200 to 890 cm^{-1} , which are likely related to the stretching

and vibrations of functional groups of various polysaccharides (Özgenç *et al.* 2017), remained even after treatment. This finding was consistent with those of a previous study (Kiyoto and Sugiyama 2022). To confirm the phenomena reported above, the absorbance ratios of the peaks were compared according to the method described by Pandey and Pitman (2003). These ratios were calculated and are listed in Table 1. These results reveal significant decreases during the desuberinization process; moreover, this occurred not only in wavenumbers related to suberin (2917 and 2849 cm^{-1}) but also in those related to lignin (1515 cm^{-1}). The decrease at 1515 cm^{-1} may be due to absorption derived from components other than lignin having increased around 1515 cm^{-1} , which would cause the 1515 cm^{-1} peak to show an apparent decrease.

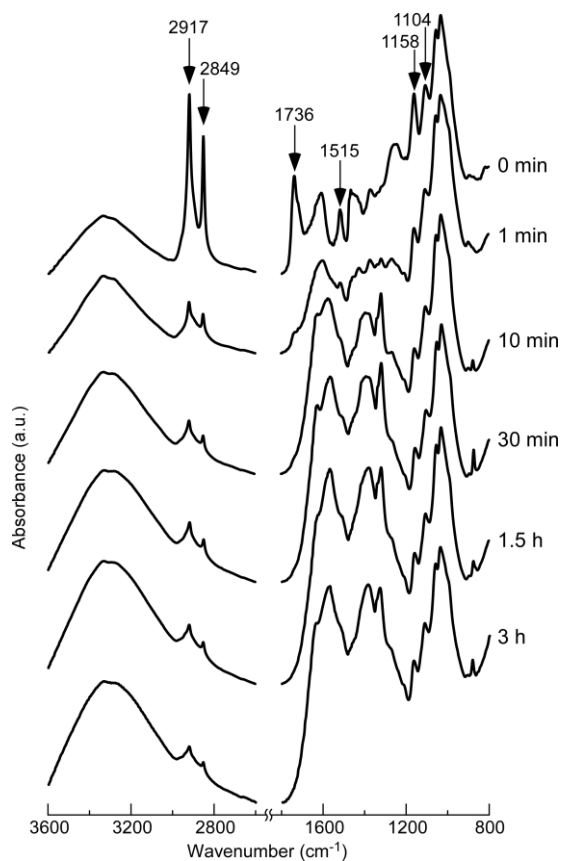


Fig. 6. Changes in the FTIR spectra of phellem specimens during desuberinization

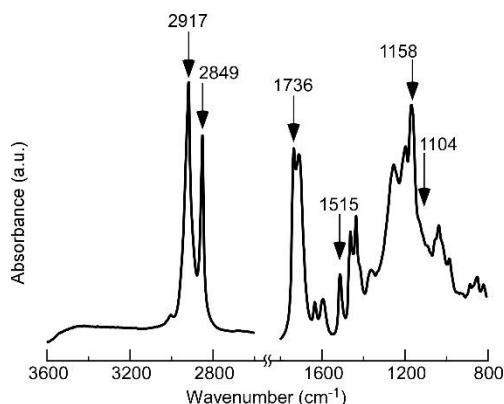


Fig. 7. FTIR spectra of suberin extracted from phellem specimens using desuberinization

Figure 8 shows typical FTIR spectra after each delignification timepoint, *i.e.*, 30 min, 6 h, 24 h, 48 h, and 96 h. It was found that the absorbance at 1515 cm^{-1} gradually disappeared with the progress of the delignification reaction. However, it should be noted that absorbances related to suberin were apparent even after 96 h of treatment. Estimated results coincided with these data, as shown in Table 1. Thus, it was concluded that delignification causes selective damage to lignin, unlike desuberinization. We believe that further verifications, such as the analysis of pore distribution based on absorption theory, are required to elucidate the full picture.

Table 1. Changes in Absorbance Ratios with Respect to I_{1104}

	Processing Time	I_{1917} / I_{1104}	I_{2849} / I_{1104}	I_{1515} / I_{1104}
	0 min	5.3	3.6	1.3
Desuberinization	1 min	1.4	0.8	0.4
	10 min	1.0	0.5	0.1
	30 min	1.3	0.6	0
	1.5 h	0.8	0.4	0
	3 h	0.8	0.3	0
	Delignification	30 min	3.3	2.2
6 h		2.9	1.9	0.2
24 h		3.4	2.3	0.1
48 h		3.2	2.1	0
96 h		2.9	2.0	0

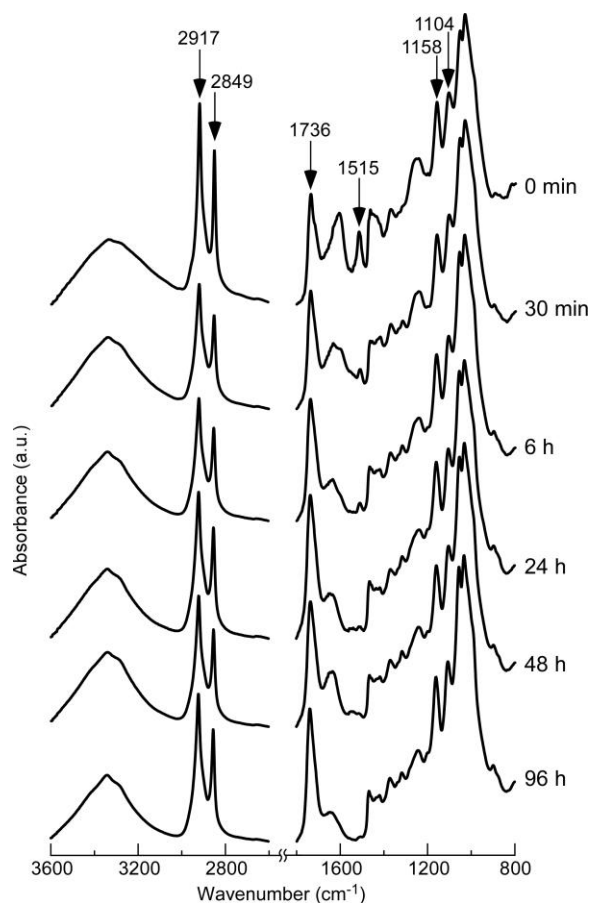


Fig. 8. Changes in the FTIR spectra of phellem specimens during delignification

CONCLUSIONS

In this study, the distribution and roles of chemical components, such as suberin and lignin, in phellem cell walls were examined. To do so, the phellem of *C. jamasakura* was used, and samples were subjected to gradual and selective removal treatments.

1. Fluorescence microscopy showed that the suberin was present locally within the secondary walls of the phellem, whereas lignin was found both in the tertiary walls and complex middle lamella (CML).
2. After gradual and selective removal treatments of suberin and lignin, it was found that removal of the suberin involved reduced levels of some matrix components, whereas delignification treatments selectively removed only lignin.
3. The gradual removal of suberin caused both loosening of phellem cells and partial collapse of secondary wall of suberin layer.
4. The presence of suberin likely contributes to the retention of the shape of phellem cell walls. This follows from the observation that the collapse of cell walls happened only following the selective removal of suberin.

ACKNOWLEDGMENTS

The authors are grateful for all the help from Wood Anatomy and Quality Laboratory members of Forestry and Forest Products Research Institute. This work was financed by a PHOENIX Grant-in-Aid.

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Article submitted: April 16, 2024; Peer review completed: May 11, 2024; Revised version received and accepted: June 3, 2024; Published: June 13, 2024.
DOI: 10.15376/biores.19.3.5129-5140