

Distribution and roles of chemical components in the phellem cell walls of *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba

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Abstract

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

Introduction

Bark affects the appearance of tree stems and usually occupies 9–15% of the stem. It consists of cellular tissues outside the vascular cambium and covers the outermost stem surface (Feng et al. 2013). 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 phellem (Shtein et al. 2023). Of these tissue types, the phellem consists of three layers with lignified and suberinized 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 cell tissues and 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 the biosynthetic using *Quercus suber* (Graça 2015; Serra and Geldner 2022). In addition, several specific mechanical properties of the phellem have been determined using *Betula platyphylla* and *Cerasus sargentii*. 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; Kiyoto and Sugiyama 2022).

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. Thus, the purpose of this study was to determine the distribution and function of key chemical components, including as suberin and lignin, in the phellem of the *Cerasus jamasakura* (Siebold ex Koidz.) H. Ohba, which is often used as a material for traditional Japanese crafts.

Materials and methods

Preparation of bark specimens

Bark specimens used were taken from the branches of *C. 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 12–14 cm. After the branches were removed using a handsaw, they were preserved in a freezer (JF-NC205F, Haier, Osaka, Japan) at –20°C.

Removal of chemical components

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

First, extractives were removed 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. We used three treatment times: 16, 20, and 48 h. After these treatments, some specimens were air-dried in an experimental laboratory, while others 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 desuberization 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 hour, and 3-hour 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) NaClO_2 (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 bark specimens and specimens subjected to the removal treatments described above were gradually dehydrated in a graded ethanol series (i.e., 45 min per step). Ethanol was removed via a graded acetone/ethanol series for 90 min 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- μm -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. Since 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 330–385 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). 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 $4,000\text{--}400\text{ cm}^{-1}$, respectively.

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, especially 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. Thus, 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. 2c, d, and e). 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. 2c). In addition, fluorescence microscopic observation showed that bright autofluorescence occurred only in areas containing secondary cells (Fig. 2d). Moreover, due to safranin staining, the tertiary walls were stained but the secondary walls were not (Fig. 2e). 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, we hypothesize 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.

FTIR analysis of the removal process of the chemical components

Figure 3 shows typical FTIR spectra before and after desuberinization, including measurements at the 1 min, 10 min, 30 min, 1.5 hour, and 3-hour 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. Therefore, most of the suberin in the phellem cell walls was likely removed by 3 h of treatment. We also found other absorbance reductions following desuberinization, including the wavenumber at 1515 cm^{-1} , likely derived from the C = C of lignin aromatics (Lopes et al. 2000; shown in Fig. 3). Over the course of the treatment we 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, we examined the absorbance ratios of peaks at 2917 , 2849 , and 1515 cm^{-1}

against a reference peak at 1104 cm^{-1} that was assigned to cellulose (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 cause of 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.

Figure 4 shows typical FTIR spectra after each delignification timepoint, i.e., 30 min, 6 h, 24 h, 48 h, and 96 h. We 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, we conclude that delignification causes selective damage to lignin, unlike desuperinization.

Microscopic observations of the removal of chemical components

Figure 5 shows the changes in autofluorescence in the phellem during the desuperinization process. We found that autofluorescence due to the existence of lignin was maintained in unstained phellem specimens even after 1.5 h of treatment (Fig. 5c). 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. 5b and c indicate areas in which lignin remains. The disappearance of fluorescence in Fig. 5e and f 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. 5b and c). These changes in phellem cell shape confirm the results of a previous study (Pereira and Marques 1988). Figure 6 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, we note that the folded tertiary walls of the phellem cell loosen after 24 h of delignification (Fig. 6b and e), after which they become thinner and aggregate (Fig. 6c and f). Thus, the differences in cell shape following desuberinization treatments suggest that suberin may maintain cell shape within the phellem.

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, we used the phellem of *C. jamasakura*, and subjected samples to selective removal treatments. After these treatments, we found that removal of the suberin involved reduced levels of some matrix components, whereas delignification treatments selectively removed only lignin. We also found that most of the suberin existed locally within the secondary walls of the phellem, whereas lignin was found both in the tertiary walls and CML. The presence of suberin likely contributes to the retention of the shape of phellem cell walls since the collapse of cell walls was observed only

following the selective removal of suberin. The knowledge acquired in this study may contribute to our understanding of the function of suberin in the phellem, although further investigation is required to determine the roles it plays.

Declarations

Author Contribution

All authors wrote the main manuscript text and figures. All authors reviewed the manuscript.

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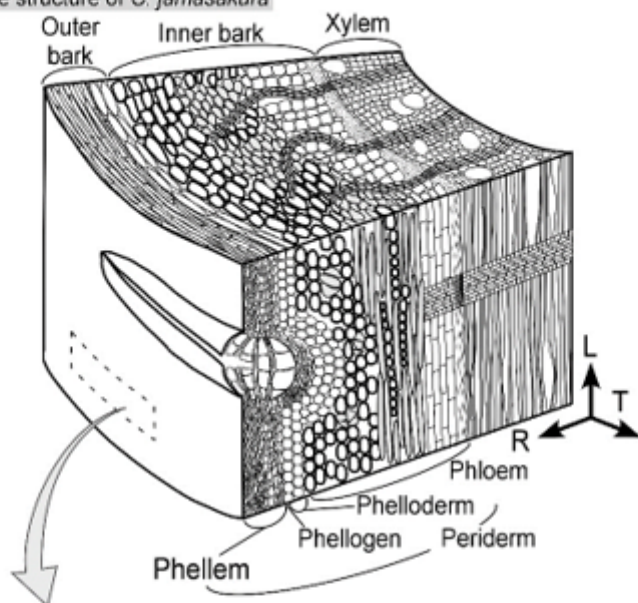
Table 1

Table 1 Changes in absorbance ratios with respect to I_{1104} .

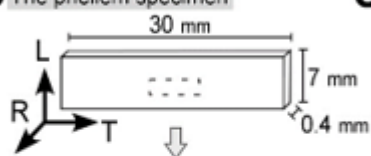
	Processing time	I_{2917}/I_{1104}	I_{2849}/I_{1104}	I_{1515}/I_{1104}
Desubерinization	0 min	5.3	3.6	1.3
	1 min	1.4	0.8	0.4
	10 min	1.0	0.5	0.1
	30 min	1.3	0.6	0
	1.5 h	0.8	0.4	0
	3 h	0.8	0.3	0
Delignification	30 min	3.3	2.2	0.4
	6 h	2.9	1.9	0.2
	24 h	3.4	2.3	0.1
	48 h	3.2	2.1	0
	96 h	2.9	2.0	0

Figures

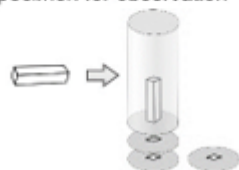
a The tissue structure of *C. jamasakura*



b The phellem specimen



The specimen for observation



Embedded in epoxy resin

c Experimental procedure

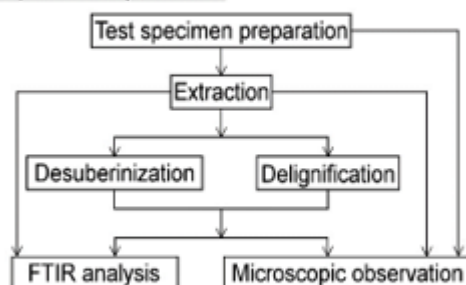


Figure 1

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

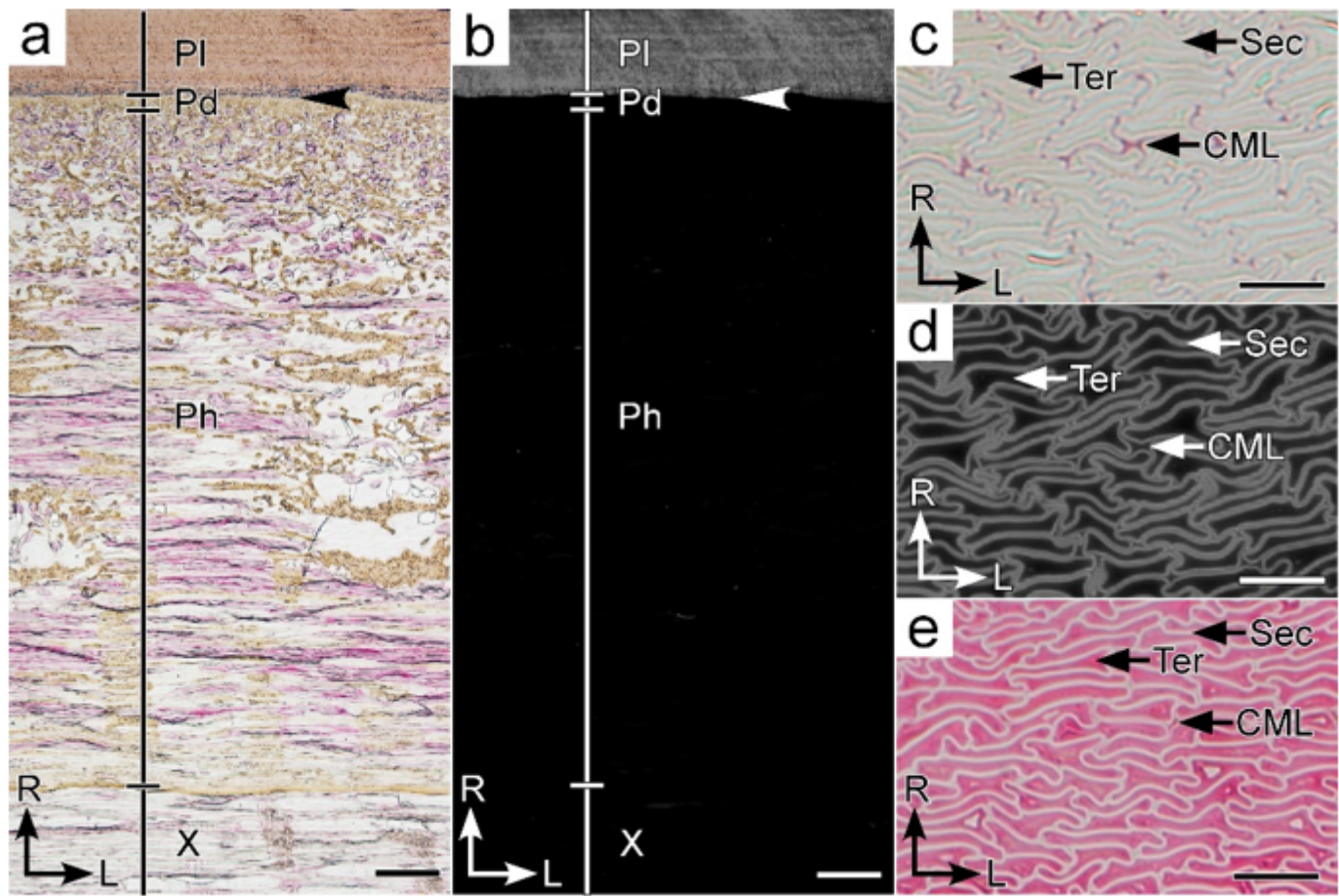


Figure 2

Radial section of bark tissue. **a** Optical microscope image of the tissue near the cambium, **b** fluorescence microscope image of the area shown in **a**, **c** and **e** optical microscope images of the phellem, and **d** Fluorescence microscope image of the area shown in **c**. Bark tissues in **a**, **b**, **c**, and **d** were stained with phloroglucinol-HCl, and the image shown in **e** image was stained with Safranin. Bars in Figs **a** and **b** indicate 200 μ m, while those in **c**, **d**, and **e** indicate 10 μ m.

Abbreviations are as follows;

PI: phellem. Arrowheads: phellogen. Pd: phelloderm. Ph: phloem. X: xylem. CML: compound middle lamella (including primary wall). Sec: secondary wall. Ter: tertiary wall.

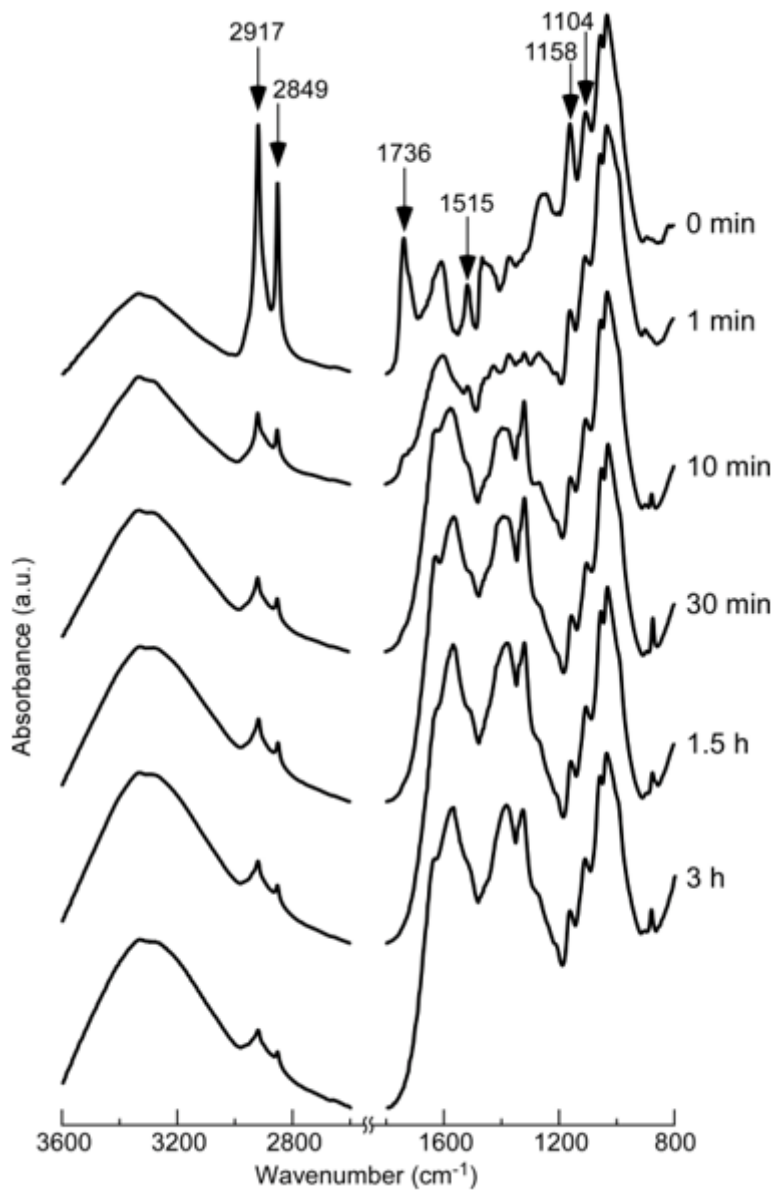


Figure 3

Changes in the FTIR spectra of phellem specimens during desperinization.

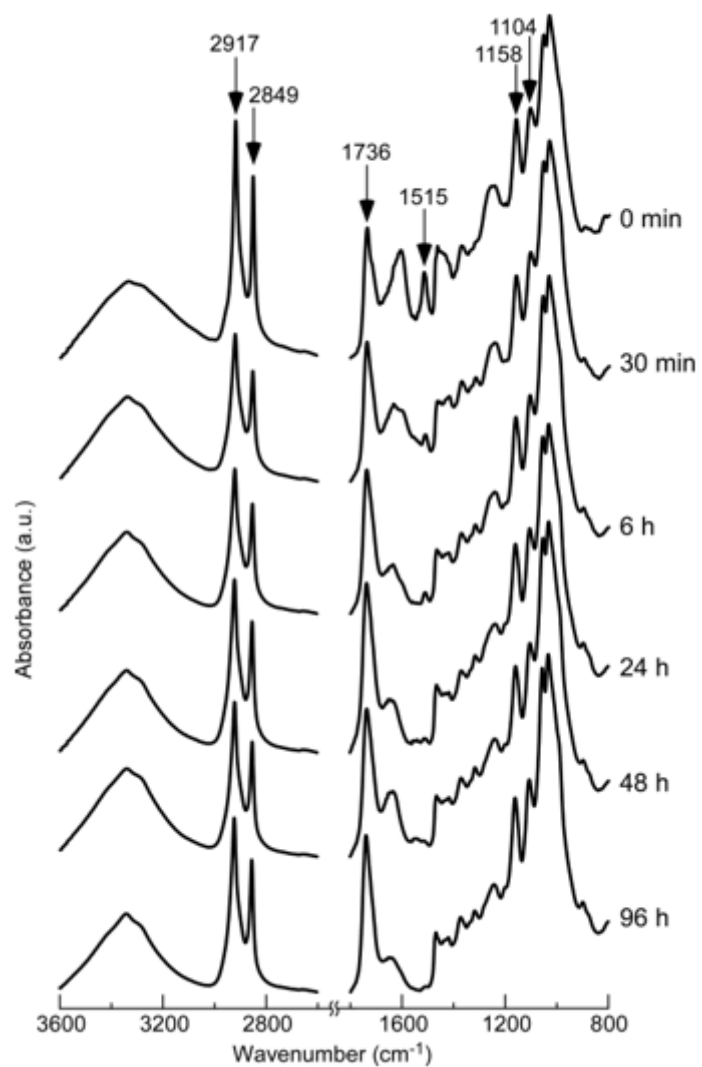


Figure 4

Changes in the FTIR spectra of phellem specimens during delignification.

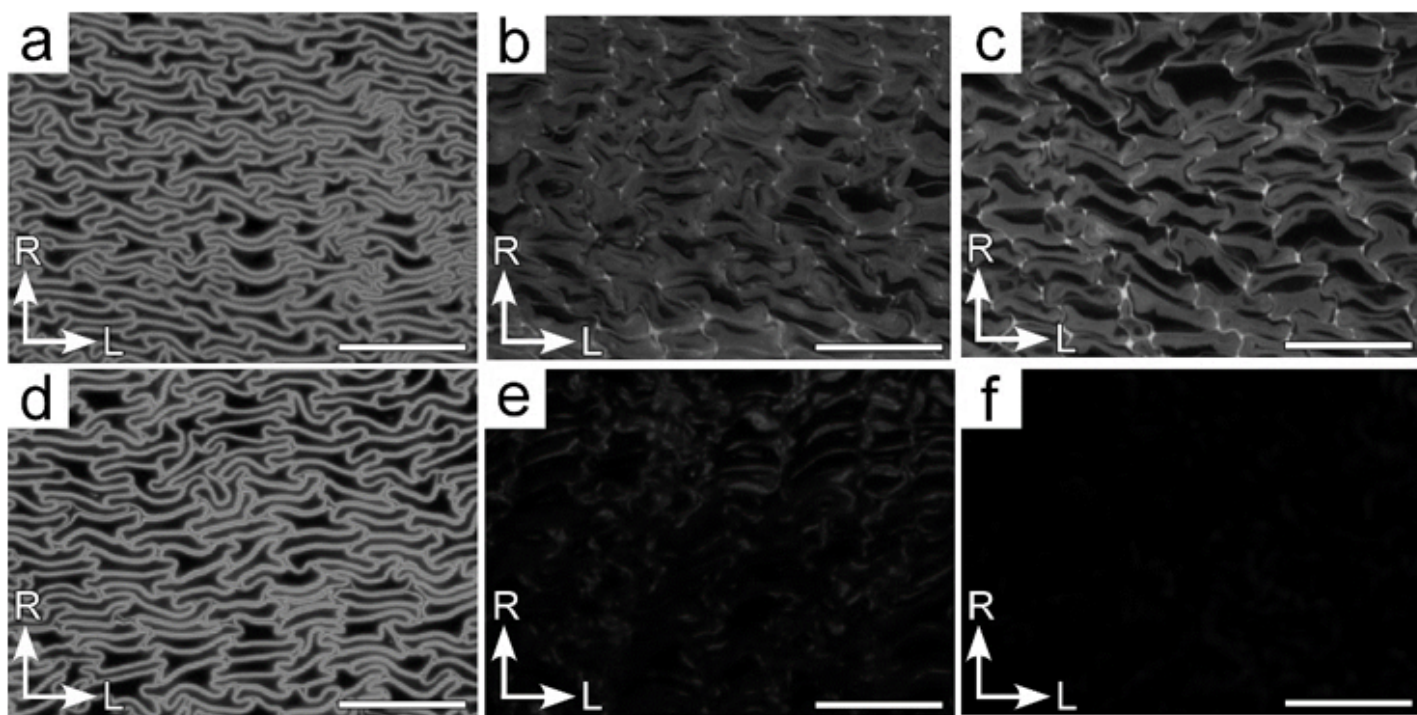


Figure 5

Temporal changes in the chemical components found in the radial section of phellem tissues during desperinization. Data obtained using fluorescence microscopy. **a–c** Unstained. **d–f** Stained with phloroglucinol-HCl. Here, specimens were treated for 0 min (**a, d**), 30 min (**b, e**), and 1.5 h (**c, f**). Bars in all figures indicate 10 µm.

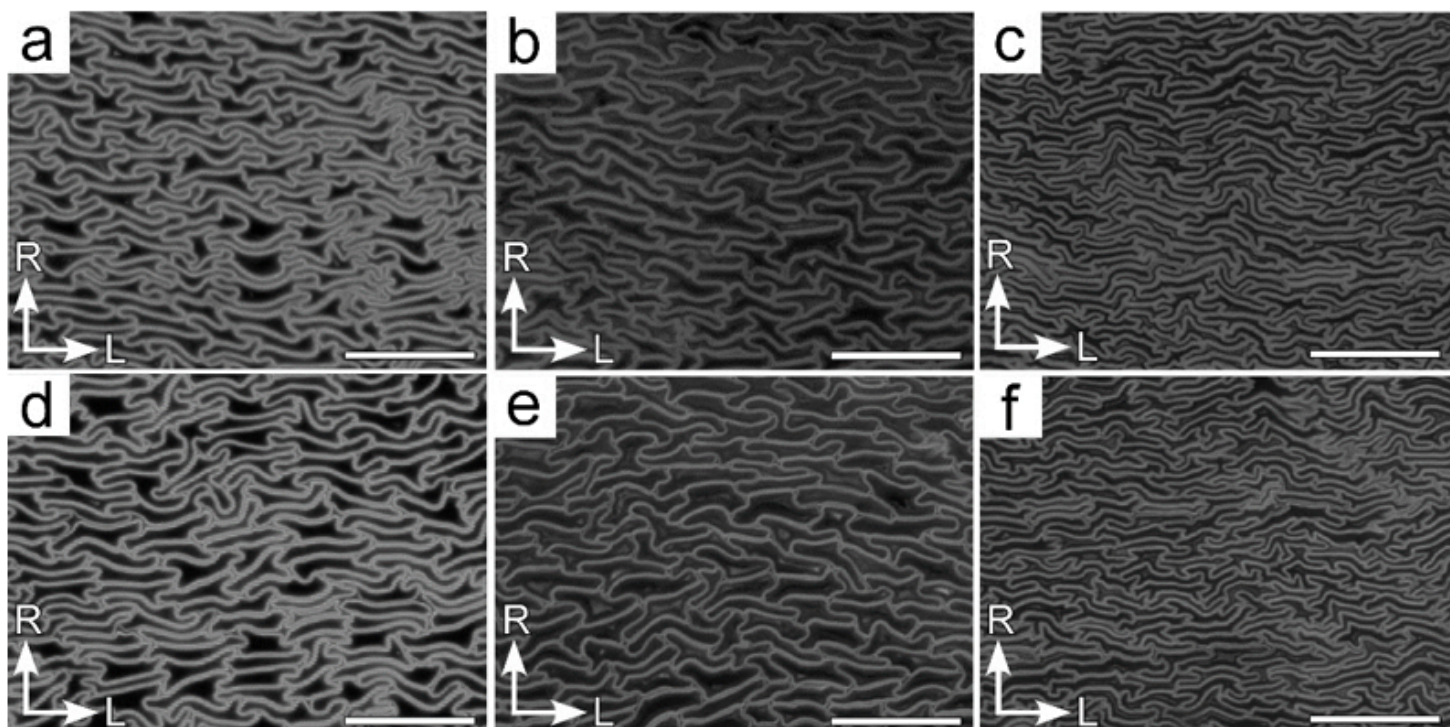


Figure 6

Temporal changes in the chemical components found in the radial section of phellem tissues during delignification. Data obtained using fluorescence microscopy. **a–c** Unstained. **d–f** Stained with phloroglucinol-HCl. Here, specimens were treated for 0 min (**a, d**), 24 h (**b, e**), and 24 h (**c, f**). Bars in all figures indicate 10 μm .