IR-SNOM Analysis of Occluding Substances in Lumina of Xylem Elements in Sapwood of *Quercus serrata* Attacked by *Platypus quercivorus*

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A new microspectroscopic technique was applied to the analysis of occluding deposits in xylem elements of *Quercus serrata*. The production of this substance is believed to be a defense response of the sapwood against fungal infection. An occluding substance about 10 μm across was analyzed by Infrared-Scanning Near-field Optical Microscopy (IR-SNOM), which allows for the measurement of IR spectrum with high spatial resolution. The near-field IR spectrum of an occluding substance was different from those of xylem elements and featured a lack of the clear C-H absorption band that should appear at 3000 – 2850 cm^{-1} . On the other hand, the absorption band of ester bond exhibited a very strong peak. Among the near-field IR spectra of related compounds, a similar ester absorption peak was observed in the spectrum of pectin and tannic acid. The presence of a C-H absorption band as a very weak peak was similar to (+)-catechin and tannic acid.

(Received November 14, 2012; Accepted January 28, 2013; Published April 10, 2013)

Introduction

When the sapwood of *Quercus serrata* is wounded by an ambrosia beetle, *Platypus quercivorus*, the area surrounding the wounded part is infected with a symbiotic fungus of this insect, *Raffaella quercivora*, and the infected area exhibits a dark color.¹ Occlusion of insoluble substances in the lumina of xylem elements appears in the circumference of the infected part, which is called reaction zone barrier.² We call these substances occluding substances, and these depositions are believed to be produced as one of the defense responses of the tree against the fungal infection.³ We conducted detailed chemical analyses of the infected sapwood and reported the following in our previous paper:⁴ 1) in the area where occluding substances were found, the lignin content obtained by the Klason method was higher, and the syringyl/guaiacyl ratio of lignin obtained by nitrobenzene oxidation and the oxygen/carbon ratio obtained by elemental analysis were lower than in the reference areas, and 2) these differences did not result from the degradation of cell wall components caused by the fungal attack but from the deposition of occluding substances produced as a defense response of the tree against fungi. Occluding substances were called “lignin-like compounds” because of their positive phloroglucinol-HCl colorization.³ Although the occluding substance has been suggested to be related to lignin,⁴ no results reported in our previous study could confirm that occluding

substances were lignin. In addition, observed differences in the analytical data between the infected area and reference area could be attributable not only to the deposition of occluding substances but also to other defense responses. In order to analyze the nature of the occluding substance itself, it is necessary to isolate the occluding substance and perform a direct analysis. However, it is difficult to extract occluding substances by organic solvents.³ Furthermore, the diameter of occluding substances is only 10 μm . Therefore we needed to directly analyze them using a microspectroscopic method.

While UV and Raman microspectroscopy have been applied to the wood tissue and cell wall, IR microspectroscopy has been seldom applied due to the low spatial resolution. Using FT-IR microspectroscopy, Kataoka *et al.*⁵⁻⁷ monitored the change of the cellulose crystalline form and orientation during cell wall formation. However, they needed a sample surface measuring more than 25 μm across to obtain the spectrum with a high enough S/N ratio to discriminate changes in these cellulose characteristics.⁷

Infrared-Scanning Near-field Optical Microscopy (IR-SNOM) can give a spatial resolution beyond the diffraction limit by applying a near-field infrared light generated on the probe tip in this apparatus.^{8,9} A near-field light is induced in an object, which is much smaller than a wavelength of irradiation light. The field size of a near-field light does not depend on the wavelength of irradiation light but on the size of the object.¹⁰ Recently, chemical imaging and spectroscopic analysis using IR-SNOM have been applied to various samples.¹¹⁻¹⁴ This analytical technique is expected to provide new topochemical information of the wood cell wall.

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Since there is no application of this IR-SNOM technique to wood samples, we tried to establish the spectroscopic IR-SNOM analysis of wood samples. We compared the near-field IR spectrum of occluding substances with those of xylem tissues and compounds related to wood components to identify the chemical constituent of occluding substances.

Experimental

Materials

A still surviving infected tree of *Q. serrata*, 11.7 m tall and 30 cm in diameter at breast height, growing at Ecohydrology Research Institute, the University of Tokyo, Japan, was cut down and a disk taken at the height of 1.4 m was frozen (in 2009). This tree was naturally wounded by *P. quercivorus* and infected by its symbiotic fungus, *R. quericivora*. A disk of wood was dried in a vacuum at room temperature and was used as a sample.

Comparison between near-field IR spectrum and usual FT-IR spectrum of standard sample

To compare the difference between the near-field IR spectrum and usual IR spectrum, milled wood lignin (MWL) was used as a standard sample. One percent solution of Birch MWL (prepared by Sugimoto *et al.*¹⁵) in $\text{CH}_2\text{Cl}_2:\text{EtOH} = 2:1$ (v/v) was prepared, and 10 μL of this solution was sprayed on the aluminum mirror. Dried samples on the Al mirror were further dried in a vacuum at 40°C overnight. This sample was subjected to spectroscopic IR-SNOM analysis and usual Fourier Transform Infrared (FT-IR) analysis.

The IR-SNOM used in this experiment was an apertureless-type near-field IR microscope (NFIR-200, JASCO Co., Ltd., Tokyo, Japan) combined with a FT-IR spectrometer (VIR-9500, JASCO Co., Ltd., Tokyo, Japan). The probe with 2 μm tip diameter was purchased from JASCO Co., Ltd. (Tokyo, Japan), and was made of optical glass fiber and coated with Au. The actual spatial resolution attained by this probe was about 2.4 μm .

The spectra were obtained in the range from 4000 to 800 cm^{-1} with an MCT detector at a spectrum resolution of 8 cm^{-1} . The scanning time was 4000 on average. Usual FT-IR measurements (transmission mode) recorded as KBr pellets were conducted under the following conditions: 64 scans, and a spectrum resolution of 4 cm^{-1} in the range from 4000 to 600 cm^{-1} . The FT-IR spectrometer used was a FT/IR-615 (JASCO Co., Ltd., Tokyo, Japan.) with a TGS detector.

Trials to obtain near-field IR spectra of an occluding substance

Small block samples (2–3 mm) were cut from the reaction zone barrier in the sapwood, and their surfaces in the radial sections were smoothed with a sliding microtome. The surface was observed by an optical microscopy (BX50, Olympus Optical Ltd., Tokyo, Japan) to find the occluding substances. After the optical microscopic observations, the probe tip of the SNOM was operated so that the top of the probe tip touched an occluding substance by monitoring the shear force signal. By this way, an occluding substance was caught on the probe tip and was analyzed by the same IR-SNOM equipment.

Spectroscopic IR-SNOM analysis of a wood fiber and ray cells

Samples of a wood fiber and the ray cells were also separated from the wood for spectroscopic IR-SNOM analysis. We made a sharp needle tip from the fine metal wire (0.35 mm diameter). Using this needle tip, samples were picked up by hand from the surface of a smoothed wood block (1–2 cm) under

Fig. 1 An example of the difference between usual FT-IR spectrum and near-field IR spectrum of MWL. Upper: Usual FT-IR spectrum of MWL measured by KBr method. Lower: Near-field IR spectrum by IR-SNOM. Sample: Birch MWL.

stereoscopic microscope observation. Each tissue was floated on the water and transferred to the pure KBr tablet. A wood fiber and the ray cells on the KBr tablet were softly pressed using a metal disc to attach the sample to the tablet.

The surface of these samples was observed by monitoring the shear force signal to obtain the topographic images surrounding the measurement point of near-field IR spectrum. No correction of these topographic images was conducted. Since the sample surface was rough, we selected the measurement points to minimize the interruption of the incident IR light from the SNOM instrument. These samples were subjected to spectroscopic IR-SNOM analysis. After the spectroscopic IR-SNOM analysis, these samples were coated with Au (20 nm thick) or Pt-Pd (10 nm thick) and subjected to SEM observations (S-4000, Hitachi Ltd., Tokyo, Japan) to identify each position of the topographic images in the samples.

Measurement of near-field IR spectrum of reference samples related to wood components

Reference samples related to wood components were subjected to spectroscopic IR-SNOM analysis to compare with the spectrum of an occluding substance. The following solution or suspension was prepared, and 10 μL of each was sprayed on the aluminum mirror: 1% solution of birch MWL (prepared by Sugimoto *et al.*¹⁵) in $\text{CH}_2\text{Cl}_2:\text{EtOH} = 2:1$ (v/v), 1% suspension of commercial birch xylan and cellulose (CF11, Whatman, Ltd., Kent, England) in $\text{CH}_2\text{Cl}_2:\text{EtOH} = 2:1$ (v/v), 2.5% commercial pectin and tannic acid in water, and 2.5% (+)-catechin in $\text{CH}_2\text{Cl}_2:\text{EtOH} = 2:1$ (v/v). Samples on the Al mirror were dried in a vacuum at 40°C, except tannic acid and (+)-catechin to which room temperature was applied, and subjected to spectroscopic IR-SNOM analysis. All the other commercial chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Results and Discussion

Figure 1 shows the near-field IR spectrum and the usual FT-IR spectrum of birch MWL as an example of the standard sample spectra. Even in the same sample, the near-field IR spectrum obtained by our IR-SNOM equipment could be different from the IR spectrum measured by the usual FT-IR in a low wavenumber region (<1300 cm^{-1}). The difference found in a low wavenumber region was mainly due to the extremely low

Fig. 2 An optical microscopic observation of an occluding substance separated onto the probe tip.

energy detected by the present IR-SNOM. Near-field IR spectrum showed a wavy baseline pattern from 2300 to 2000 cm^{-1} , which was previously mentioned by Kebukawa *et al.*¹³ This is thought to be due to interference of IR light scattered on the sample surface and reflected from the Al mirror.

At this moment, it is impossible to isolate the occluding substances in an amount enough to conduct direct chemical analyses. Therefore, first we tried to develop a method for spectroscopic analysis of the wood tissue.

Considering the small diameter of the occluding substances, we selected spectroscopic IR-SNOM analysis as the method. Because the chemical components of each xylem tissue are different, each wood tissue has to be separated from surrounding tissues before subjecting to spectroscopic IR-SNOM analysis in order to avoid possible interference from the surrounding tissues. In order to separate the occluding substance, the probe tip of the SNOM was operated so that it touched an occluding substance by monitoring the shear force signal of the IR-SNOM equipment. In this way, an occluding substance was caught on the probe tip (Fig. 2) and was analyzed by the same IR-SNOM equipment. Spectrum (A) of Fig. 3 represents that of an occluding substance on the probe tip and (D) of Fig. 3 shows the usual FT-IR spectrum obtained from wood powder prepared from the xylem of *Q. serrata*. Spectrum (A) was quite different from spectrum (D). Although the very strong peak of ester bond was seen in spectrum (A) (1750 cm^{-1}), the C-H absorption band that should appear at 3000–2850 cm^{-1} was hardly seen.

A wood fiber and ray cells were also separated from the smooth surface of a wood block, and subjected to spectroscopic IR-SNOM analysis. The images of a wood tissue and ray cells on the pure KBr tablet are shown in Figs. 4 and 5. The wide topographic images of Figs. 4(b) and 5(b) obtained from SNOM seemed to correspond to the square of SEM images (Figs. 4(a) and 5(a)), respectively. As shown in Figs. 4(c) and 5(c), fine topographic images corresponded to the black square of (b) in each respective figure. From SEM observations, the lumen of wood fiber seemed to be exposed by the sample preparation (Fig. 4(a)), and a part of ray cells cut by microtome seemed to remain on the adjacent ray cell (Fig. 5(a)). Further instrumental improvement of SNOM might make these images clearer.

From the area in (c) of Figs. 4 and 5, we could obtain

Fig. 3 IR spectrum and near-field IR spectra. A, occluding substance on the probe (scan time 16000); B, wood fiber on the KBr tablet; C, ray cell on the KBr tablet; D, wood powder (usual IR spectrum); E, birch MWL on the Al mirror; F, apple pectin on the Al mirror; G, birch xylan on the Al mirror; H, cellulose (CF11) on the Al mirror; I, catechin on the Al mirror; J, tannic acid from Chinese natural gall nuts on the Al mirror. Scan time was 4000 except for B and D.

near-field IR spectra of a wood fiber and ray cells, respectively. As noted previously, at lower wavenumbers, the pattern of near-field IR spectra obtained from a wood fiber (Fig. 3B) and ray cells (Fig. 3C) differed from the usual IR spectrum obtained from wood powder (Fig. 3D). However, although the near-field IR spectra of (B) and (C) were obtained by the same IR-SNOM equipment, these patterns were also slightly different in the same wavenumber region. These results indicate that spectrum (B) and (C) represent structural characteristics peculiar to each tissue. Since the spectral pattern of wood tissue such as (B) and (C) were clearly different from that of (A), occluding substances had different chemical components from these wood tissues.

The spectra of (E) to (J) in Fig. 3 were obtained from compounds related to wood components. Birch MWL (E) was used as a lignin sample, and apple pectin (F), birch xylan (G), and cellulose (H) were used as the sample of polysaccharide in the wood cell wall. Catechin (I) and tannic acid (J) were used as the sample of hard wood extractives. Among them, the absorbance band due to ester bond (1750 cm^{-1}) exhibited in the spectrum of an occluding substance (A) was seen in the spectrum from pectin (F). Since occluding substances in the section were stained with not only phloroglucinol-HCl but also ruthenium red,³ we considered the possibility of the existence of pectin as one constituent of the occluding substances. Figure 6 shows the magnified spectra of an occluding substance (A),

Fig. 4 SEM image and topographic image of a wood fiber. (a) SEM image of a wood fiber on the pure KBr tablet. The sample was coated with Au. (b) Wide topographic image: corresponds to the black square of (a). This image was taken at a resolution of 20×20 pixels. (c) Fine topographic image: corresponds to the black square of (b). This image was taken at a resolution of 256×128 pixels. The lighter colors of (b) and (c) correspond to positions higher than the black ones.

Fig. 5 SEM image and topographic image of ray cells. (a) SEM image of ray cells on the pure KBr tablet. (b) Wide topographic image: corresponds to the black square of (a). This image was taken at a resolution of 20×20 pixels. (c) Fine topographic image: corresponds to the black square of (b). This image was taken at a resolution of 256×128 pixels. The lighter colors of (b) and (c) correspond to positions higher than the black ones.

Fig. 6 Comparison of magnified near-field IR spectra. A, F, I, and J: See Fig. 3. Square: represents wavenumber region about 1750 cm^{-1} . Square dotted line: represents wavenumber region from 3000 to 2850 cm^{-1} .

apple pectin (F), (+)-catechin (I), and tannic acid (J). As shown in Fig. 6, the ester peak of tannic acid (J) was slightly different (1720 cm^{-1}) from that of an occluding substance (A). Spectra of (+)-catechin (I) and tannic acid (J) did not show clear peaks due to the C-H absorption band (3000–2850 cm^{-1}), which was similar to the spectrum of occluding substances (A) (Fig. 6). Although the occluding substances are called “lignin-like compounds”,³ the near-field IR spectrum was not similar to that of lignin (E) but resembles that of tannic acid (J). None of the single compounds examined here could satisfy the structural feature of occluding substances implied by its near-field IR spectrum.

With the use of the present IR-SNOM equipment, it was difficult to obtain near-field IR spectral mapping from these samples because the measurement time per one spectrum was too long to ensure good measurement. In order to conduct near-field IR spectral mapping from wood samples, it is necessary to obtain the spectrum with a high *S/N* ratio in a shorter measuring time. Further improvement of the present sample preparation and IR-SNOM equipment would provide new topochemical information of the wood cell wall.

Conclusions

Near-field IR spectroscopy was applied to the analysis of occluding substances deposited in the lumina of xylem elements infected by the fungus *R. quercivora*. This was the first application of this spectroscopic method to wood components. Near-field IR spectrum of an occluding substance featured the presence of a strong ester bond peak and the absence of a clear C-H bond peak. It was presumed that occluding substances are not present as a pure compound but as a mixture of different types of compounds.

Acknowledgements

This work was supported by Grant-in-Aid for JSPS Fellows. The authors thank Ecohydrology Research Institute, the University of Tokyo, Japan, for providing the sample of *Q. serrata* wounded by *P. quercivorus*.

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