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Kwang Ho Lee · Seung Gon Wi · Adya P. Singh
Yoon Soo Kim

Micromorphological characteristics of decayed wood and laccase produced by the brown-rot fungus *Coniophora puteana*

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Abstract Microscopic examination showed the cell wall decay pattern produced by the brown-rot fungus *Coniophora puteana* to be different from the degradation pattern known to be typical for brown-rot fungi. Erosion and thinning of cell walls in patterns considered to be characteristic of white-rot decay were observed. In particular, the fungal strain COP 20242 degraded secondary cell wall layers extensively, and also degraded lignin-rich middle lamellae. Some strains of *C. puteana* produced soft-rot type cavities in the S2 layer. All strains of *C. puteana* employed in the present work showed a positive reaction to tannic acid in the Bavendamm test, indicating the production of laccase. Microscopic and enzymatic studies provided evidence to suggest that the wood decay by *C. puteana* is unique both in terms of micromorphological and enzymatic patterns of cell wall degradation. This is because brown-rot fungi are not generally known to form cavities in the cell walls or to produce lignin-degrading enzymes. These observations suggest that lignin degradation capacity of brown-rot fungi may be greater than previously considered.

Key words Brown-rot fungus · *Coniophora puteana* · Micromorphology · Laccase · Lignin degradation

Introduction

Brown-rot decay of wood is characterized by rapid degradation of the S2 layer, but the S3 layer and lignin-rich middle lamellae appear to resist degradation.[1–3] During brown-rot decay, cellulose and hemicelluloses are rapidly and extensively depolymerized, but degradation of lignin is limited.[4] Compared with white-rot fungi, little is known about the lignin-degrading capacity of brown-rot fungi, except for a few reports on the presence of ligninolytic enzymes in brown-rot fungi.[5–7]

In earlier work, Kim et al.[8] observed that the brown-rot fungus *Coniophora puteana* degraded hardwood cell walls, including middle lamellae, in a manner characteristic of white-rot decay. Kleist and Schmitt[9] recently observed the soft rot-like decay pattern in hardwood produced by *C. puteana*. These modes of degradation are different from the pattern known to be typical for brown-rot decay, and this suggests that some brown-rot fungi can also substantially degrade lignin.

The present work was undertaken to compare softwood and hardwood species with respect to the patterns of degradation produced by *C. puteana*, using four different strains of this fungus. In addition, the production of laccase, one of the major ligninolytic enzymes, in *C. puteana* was investigated because brown-rot fungi are not generally known to produce lignin-degrading enzymes. The effect of different carbon sources on the activity of laccase was also examined. We describe here two different decay patterns produced by the brown-rot fungus *C. puteana* and provide evidence that this fungus produces laccase, an enzyme considered to play an important role in lignin biodegradation.

Materials and methods

Fungal strains

Four fungal strains of *Coniophora puteana* (Schumacher: Fries) Karsten obtained from four different sources were used in the present work: (1) *C. puteana* IMSUN 31002 from the Institute of Microbiology at Seoul National University, Korea; (2) *C. puteana* KCTC 6720 (Schumacher) Karsten from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) in Daejeon, Korea; (3) *C. puteana* COP 20242 from the Forestry Research Institute in Seoul, Korea; and (4) *C. puteana* RLG-6858 from the Forest Products Laboratory, USDA Forest Service, Madison, USA.

K.H. Lee · S.G. Wi · A.P. Singh · Y.S. Kim (✉)
Department of Forest Products and Technology, Chonnam National University, Yongbong 300, Gwangju 500-757, Korea
Tel. +82-62-530-2093; Fax +82-62-530-2099
e-mail: kimys@chonnam.ac.kr

Microscopy of degraded wood

Blocks of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) and oak (*Quercus accutissima* Carr.) wood were incubated with four different *C. puteana* strains using the standard soil block procedure.[10] Wood blocks (15 × 15 × 5 mm) of pine and oak were placed on a feather slip of birch wood. After a 12-week incubation, wood samples for microscopic observations were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). Sections were observed with a light microscope (Zeiss Axiolab), and a confocal laser scanning microscope (CLSM; Leica TCS MT) using lignin autofluorescence. For scanning electron microscopy (SEM), samples were freeze-dried, and then gold-coated prior to examination with a Hitachi S-2400 SEM.

Bavendamm test

To confirm laccase production, *C. puteana* was cultured at 22°C for up to 3 weeks in potato–dextrose–agar (PDA) medium containing 0.5% tannic acid.[11]

Assay of laccase activity

A liquid culture medium was prepared for the determination of laccase activity.[12] Flasks (500 ml), containing 100 ml media, were inoculated with five agar plugs (1 cm^2 each) derived from well-grown petri dishes. Modified nitrogen-limited basal medium[12] was then applied, which contained (per liter) 10 g glucose, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 4.4 g 2,2-dimethylsuccinic acid, 0.11 g diammonium tartrate, 10 ml mineral stock solution, and 1.5 ml vitamin stock solution. To monitor the influence of different carbon sources on the production of laccase, sawdust from pine or oak wood (40–60 mesh size, 5 g/l), was placed in flasks containing liquid culture medium and the pH was adjusted to 4.5 before autoclaving. The flasks were incubated at 22°C for up to 10 days without shaking. For the determination of extracellular enzyme activity, samples of the culture fluids were taken daily, and cell-free culture filtrates were obtained by filtration using disposable filter holders (0.2 mm, Schleicher and Schüll, Dassel, Germany).

Laccase activity in the liquid culture was determined by the method of Galliano et al.[13] The enzyme activity was assayed by measuring the absorbance of syringaledazine at 526 nm. One unit of laccase activity was defined as the amount of laccase that oxidized 1 μmole of the syringaldazine per minute. For control, the absorbance of only the liquid medium was measured.

Results and discussion

Decay pattern of *Coniophora puteana* strains

Coniophora puteana degraded the tracheids and the fibers, while vessel walls remained intact. Pine and oak woods attacked by *C. puteana* strains showed two different decay patterns: cavity formation in the S2 layer, and extensive degradation of cell walls, including the lignin-rich middle lamellae without cavity formation. Cavities were produced in the S2 layer of softwood tracheids as well as in the hardwood fibers by the fungal strains *C. puteana* IMSUN 31002 (Fig. 1), *C. puteana* KCTC 6720 (Fig. 2). The cavities were aligned along the orientation of the cellulose microfibrils (Fig. 2).

Some fungal strains of *C. puteana* degraded the wood cell walls from the lumen toward the middle lamellae (Figs. 3, 4). In particular, *C. puteana* COP 20242 uniformly degraded all cell wall layers, including the lignin-rich middle lamellae

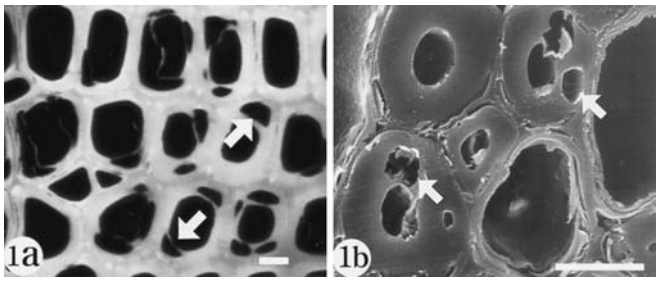

Fig. 1. Transverse sections of pine wood (**a**) and oak wood (**b**) decayed by *Coniophora puteana* IMSUN 31002 after 12-week incubation. Note the cavities (*arrows*) in the S_2 layer both in tracheids and fiber cell walls. *Bars* 10 μm

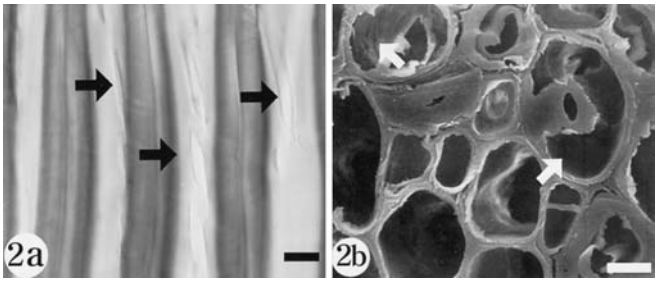

Fig. 2. Longitudinal sections of pine wood (**a**) and transverse section of oak wood (**b**) decayed by *Coniophora puteana* KCTC 6720 after 12-week incubation. Note the cavities are aligned along the orientation of cellulose microfibrils in the tracheid wall (**a**). The S_2 layer of the fiber wall is extensively degraded (*arrows*). *Bars* 10 μm

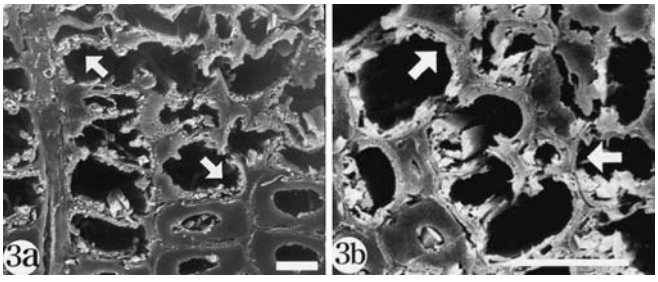

Fig. 3. Transverse sections of pine wood (**a**) and oak wood (**b**) decayed by *Coniophora puteana* RLG-6858 after 12-week incubation (SEM). Note severe degradation of cell walls in both softwood tracheids and hardwood fibers (*arrows*). *Bars* 20 μm

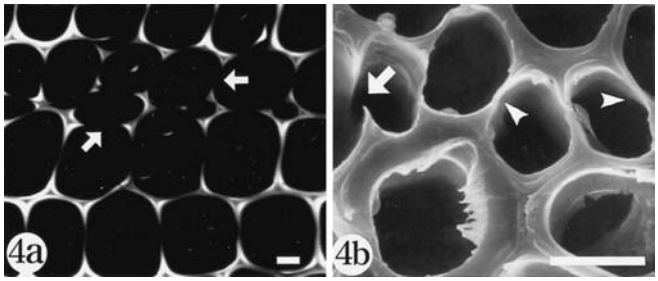

Fig. 4. Transverse sections of pine wood (**a**) and oak wood (**b**) decayed by *Coniophora puteana* COP 20242 after 12-week incubation. Note the erosion (*arrowheads*) of cell walls and degradation of middle lamellae (*arrows*). *Bars* 10 μm

Table 1. Reaction of Bavendamm test by *Coniophora puteana* strains

Strains	After 20 days of incubation
C. puteana IMSUN 31002	Strong
C. puteana KCTC 6720	Mild
C. puteana RLG-6858 sp.	Weak
C. puteana COP 20242	Strong

(Fig. 4). This decay pattern resembled the simultaneous degradation of wood cell walls caused by white-rot attack, in which polysaccharides and lignin are removed more or less at the same time.[3] Complete cell wall degradation was found not only in hardwood fibers but also in softwood tracheids, suggesting that both guaiacyl and syringyl lignin can be degraded by fungal strains of *C. puteana*.

According to Highley et al.,[2] there are two physiological types of hyphae in brown-rot fungi. Some have the capacity to degrade and metabolize all cell wall compounds, including lignin, while other hyphae merely modify lignin.[2] Evidence of complete cell wall degradation by *C. puteana* observed in our work supports the suggestion of Highley et al.[2] that some brown-rot hyphae can degrade all cell wall components. Further studies are needed to screen the lignin-degrading capacity of other brown-rot fungi.

Laccase activities of *Coniophora puteana* strains

Degradation of middle lamellae and secondary cell walls by *C. puteana* implies that enzymes or agents responsible for the degradation of lignin must be present in the brown-rot fungus *C. puteana*. All *C. puteana* strains examined in the present work showed positive reactions to tannic acid used in the Bavendamm test. The data from the Bavendamm test in Table 1 provide evidence for the presence of laccase activity in this fungus.

Because there are no detailed reports on laccase activity by *C. puteana*,[14] the production of laccase activity by the fungus was monitored in the liquid culture. Maximum laccase activity by most of strains was observed after 3–5 days of incubation, after which the level of activity dropped (Fig. 5). Among the strains tested, *C. puteana* IMSUN 31002 produced higher amounts of laccase than those produced by other strains when hardwood sawdust of oak was added to the liquid culture medium (Fig. 6). A similar increase in laccase activity was also detected in the culture medium of *C. puteana* IMSUN 31002 mixed with softwood sawdust of Japanese red pine (activity not shown). The reason for the increase in laccase activity after addition of sawdust remains unclear.

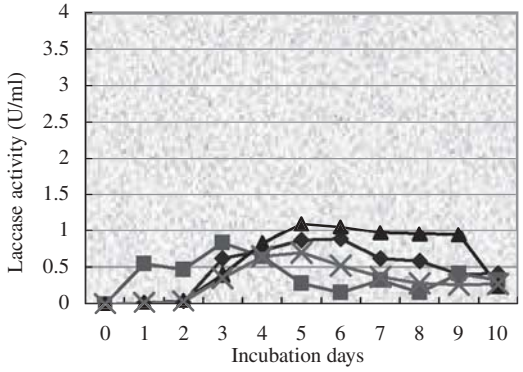

Fig. 5. Laccase activity in liquid cultures during the growth of *Coniophora puteana* strains. *Diamonds*, *C. puteana* KCTC 6720; *squares*, *C. puteana* COP 20242; *triangles*, *C. puteana* IMSUN 31002; *crosses*, *C. puteana* RLG 6858

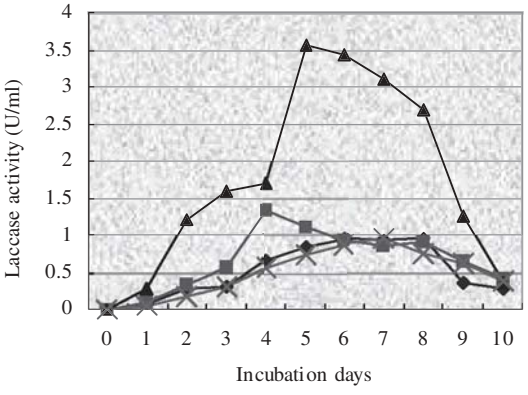

Fig. 6. Laccase activity in liquid cultures with sawdust of oak during the growth of *Coniophora puteana* strains. Symbols are the same as in Fig. 5

Compared with white-rot fungi, little is known about lignin-degrading enzymes of brown-rot fungi, except for a few reports that indicate the presence of lignin peroxidase[6] and manganese peroxidase[5] in brown-rot fungi. Laccase activity has been also demonstrated in some brown-rot fungi, such as *Gloeophyllum trabeum*, *Postia placenta* Mad 757, *Wolfiporia cocos*, *C. puteana*, and *Merilus lacrymans*.[7,14,15] The nucleotide sequence of *G. trabeum* deduced from polymerase chain reaction primers showed high similarity (70% to 81%) to the corresponding amino acid sequences deduced from laccase gene sequences of typical white-rot fungi such as *Trametes versicolor*, and *Lentinula edodes*.[7]

Our study has provided further support for the presence of laccase in the brown-rot fungus *C. puteana*, in addition to providing micromorphological evidence that this fungus has the capacity to degrade all wood cell wall components.

Understanding the biochemistry and physiology of the degradative system employed by brown-rot fungi for lignin degradation is a current challenge. Kirk[16] proposed involvement of extracellular oxygenase in lignin decomposition by brown-rot fungi. It is curious that *C. puteana* COP 20242 with relatively low activity of laccase (Fig. 6) showed a capacity to extensively degrade all cell wall layers including the middle lamellae (Fig. 4). This leads us to assume that in addition to laccase, *C. puteana* may employ other degradative systems or enzymes for lignin decomposition. Further studies are necessary to determine the involvement of enzymes such as lignin peroxidase and manganese peroxidase.

Kim et al.[17] cytochemically demonstrated the production of hydrogen peroxide in the *C. puteana* hyphae. Hydrogen peroxide is required for the catalytic reaction of lignin-degrading enzymes such as lignin peroxidase.[3] In combination with iron, hydrogen peroxide has also been considered to play a key role in brown-rot fungal decomposition of cellulose.[18] Whatever the origin of hydrogen peroxide in *C. puteana*, it cannot be ruled out that reactive oxygen species produced by *C. puteana* are involved in some way in lignin degradation.

In conclusion, on the basis of enzyme assays and micromorphological observations of wood cell wall degradation by *C. puteana*, the present study has provided evidence for degradation of all cell wall layers, including lignin-rich middle lamellae, and for the production of laccase activity in this fungus. We suggest that brown-rot fungi may have a greater capacity to degrade lignin than previously thought. Molecular approaches, such as those needed to identify species and isolates of *Coniophora* cellar fungi,[19] will no doubt prove useful in identifying potentially important wood-degrading fungi of unknown identity and in characterizing the mode of their action.

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