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Microscopic Patterns of Decay caused by *Tyromyces palustris* and *Gloeophyllum trabeum* in Korean Red Pine and Radiata Pine Woods*1

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ABSTRACT

The objective of this study was to elucidate the microscopic patterns of decay caused by brown-rot fungi of *Tyromyces palustri* and *Gloeophyllum trabeum* in Korean red pine (*Pinus densiflora*) and radiata pine (*Pinus radiata*) woods through light and electron microscopies. The ultrastructural changes of cell walls attacked by the two brown-rot fungi were compared in this respect. Macroscopically, radiata pine showed more ring and radial checks than Korean red pine. Microscopically, with the progress of decay, spiral checks associated with cross-field pits and bore holes in the cell wall were more remarkably numerous in the radiata pine than in the Korean red pine. In the radiata pine, *G. trabeum* produced more spiral checks in the cell wall than *T. palustris*. In the advanced stages of decay by *G. trabeum*, the erosions of ray cell walls were identified both in the Korean red pine and radiata pine but S_3 layers of tracheid walls were eroded only in the Korean red pine.

Keywords : Korean red pine (*Pinus densiflora*), radiata pine (*Pinus radiata*), brown-rot fungi, *Tyromyces palustris*, *Gloeophyllum trabeum*, decay stage, anatomical characteristics, cell wall, ultrastructural change, light microscopy, scanning electron microscopy, transmission electron microscopy

1. INTRODUCTION

In the favorable conditions, microorganisms invade and degrade wood in use, thereby causing great losses. Each particular microorganism has its unique pattern of wood deterioration. Earlier studies on microscopic changes of wood structure attacked by microorganisms have been thoroughly reviewed by Wilcox (1970).

The most serious kind of microbiological deterioration of wood is caused by basidiomycetes, including brown-rot and white-rot fungi, because they can cause the wood structure to fail rapidly (Green and Highley 1997).

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White-rot fungi are able to fragment the major structural polymers of cellulose, hemicellulose, and lignin in the cell wall of hardwood and softwood at approximately equal rate. Thus, these white-rot fungi cause the wood to become paler in color and eventually reduce it to a fibrous, whitish mass. Differently from white-rot fungi, brown-rot fungi can preferentially attack cellulose and hemicellulose components of cell walls but leave lignin essentially undegraded, albeit lignin is modified by demethylation and hydroxylation. Therefore, the wood degraded by the brown-rot fungi consists of an amorphous, crumbly brown residue that is composed primarily of lignin (Eriksson *et al.* 1990; Goodell *et al.* 1997; Carll and Highley 1999).

In the recent researches using electron microscopy, however, ultrastructural changes of wood by brown-rot fungi indicated that substantial degradation of lignin occurred in some cases (Messner and Stachelberger 1984; Highley *et al.* 1985). Also, brown-rot fungi were reported to be able to produce some hyphae capable of degrading lignin (Highley *et al.* 1985).

Moreover, it was noted that in the brown-rotted wood, unlike white-rotted, the progressive cell wall thinning did not occur but the cell walls were degraded very irregularly (Cowling 1961; Wilcox 1968). This uneven degradation of cell wall was reported to be dependent upon the resistance to decay between the cell wall layers by intricate relationships between dense of the cell wall, cellulose crystallite, lignin content, and chemical composition. Especially, the cell wall degradation was thought to be greatly related to content of lignin, the most decay-resistant component to microorganism (Wilcox 1970; Kuo *et al.* 1988). In recent studies, however, it was raised that the degradation pattern varied according to lignin/polysaccharide linkages, different basic units, and proportion consisting of lignin as well as lignin content (Hudson 1986).

Understanding how brown-rot fungi degrade wood has gained increasing attention in recent years. Despite these substantial efforts, however, the mechanisms of penetrating wood cell walls and degrading cellulose by brown-rot fungi are still not clear. Therefore, studies on the ultrastructural changes of cell walls in the progressive decay by brown-rot fungi through different advanced techniques will have an influence on the overall comprehension of pattern of wood decay and will contribute to the technical development for the effective preservation of wood.

In this relation, this study is carried out to demonstrate the patterns of decay caused by brown-rot fungi of *Tyromyces palustris* and *Gloeophyllum trabeum* in the Korean red pine and radiata pine woods through light and electron microscopies.

2. MATERIALS and METHODS

2.1. Materials

Korean red pine (*Pinus densiflora*) and radiata pine (*Pinus radiata*) sapwood blocks measuring 19 × 19 × 19 mm were prepared from air-dried boards. Two brown-rot fungi, *Tyromyces palustris* and *Gloeophyllum trabeum*, were used as test fungi. *T. palustris* is the brown-rot representative in the Korean Industrial Standard (KS), the Japanese Industrial Standard (JIS), and the Japanese Agricultural Standard (JAS) for appraising wood preservatives. And *G. trabeum* is one of the brown-rot representatives in standards of the American Society for Testing and Materials (ASTM) and the American Wood-Preservers' Association (AWPA) for appraising wood preservatives and for evaluation natural decay resistance.

2.2. Methods

2.2.1. Preparation of Decayed Blocks

Test blocks were oven-dried at 55°C for 24 hours, weighed to obtain the initial dry weight, and sterilized at 121°C for 30 minutes. These blocks were exposed to *T. palustris* and *G. trabeum* following the soil-block method as described in ASTM D2017-81 (ASTM 1994).

2.2.2. Microscopic Studies

2.2.2.1. Light Microscopy (LM)

Small specimens, $1 \times 1 \times 1$ cm^3, cut from decayed wood blocks were boiled in an autoclave for softening. Transverse, radial, and tangential sections were sliced with a sliding microtome, and stained with aqueous safranin. They were immediately dehydrated and cleared in the ethanol and xylene series, followed by mounting with Canada balsam.

Also, small specimens of $1 \times 1 \times 1$ cm^3 were prepared from decayed wood blocks and were immediately fixed with a FAA as described earlier (Kuo *et al.* 1988). They were rinsed, dehydrated in the ethanol and xylene series, and embedded in paraffin wax. The sections cut by a sliding microtome were double-stained and were finally mounted after dehydration in the above-mentioned ethanol and xylene series (Wilcox 1964).

2.2.2.2. Scanning Electron Microscopy (SEM)

FAA-fixed specimens were treated with a PEG-1500 as described earlier (Wilcox 1964). Transverse, radial, and tangential surfaces of embedded specimens were prepared by a razor-blade cutting technique (Exley *et al.* 1974). Cubic specimens containing razor-blade- cut surfaces were removed from the mounting blocks, washed with several changes of warm distilled water to remove the embedding matrix (Wilcox 1964), and dried in a drying oven at 103°C. Dried specimens were mounted on SEM stubs, coated to approximately 300 Å thick with gold-palladium in a sputter coater, and examined with a JEOL-JSM 5410 at 25 kV.

2.2.2.3. Transmission Electron Microscopy (TEM)

Small specimens of $1 \times 1 \times 1$ mm^3 from decayed wood blocks were prepared as the method described above (Eom and Butterfield 1997; NICEM 2003). Semi-thin sections were cut with a diamond knife using a ultramicrotome and stained with toluidine blue for light microscopy. Finally, thin sections were cut with a diamond knife, double-stained (Weakley 1972; Sato 1968), and viewed with a JEOL-JEM 1010 transmission electron microscope at 80 kV.

3. RESULTS and DISCUSSION

3.1. Macroscopic Feature

Generally, the wood blocks attacked by *T. palustris* showed ring checks but those by *G. trabeum* exhibited radial checks (Fig. 1). And these ring and radial checks were more prominent in the radiata pine than in the Korean red pine.

3.2. Microscopy

Because of its generally soft or friable nature, decayed wood was considered a refractory specimen in the preparation of sliced sections for microscopic examination. Cutting across the grain from the decayed wood blocks generally produced considerable distortion. Thus, small decayed specimens for microscopic observation were immediately fixed with FAA. After removal of the fixative with several changes of distilled water, specimens for LM were embedded in paraffin, sectioned, and then double-stained by a picro aniline blue method (Wilcox 1964).

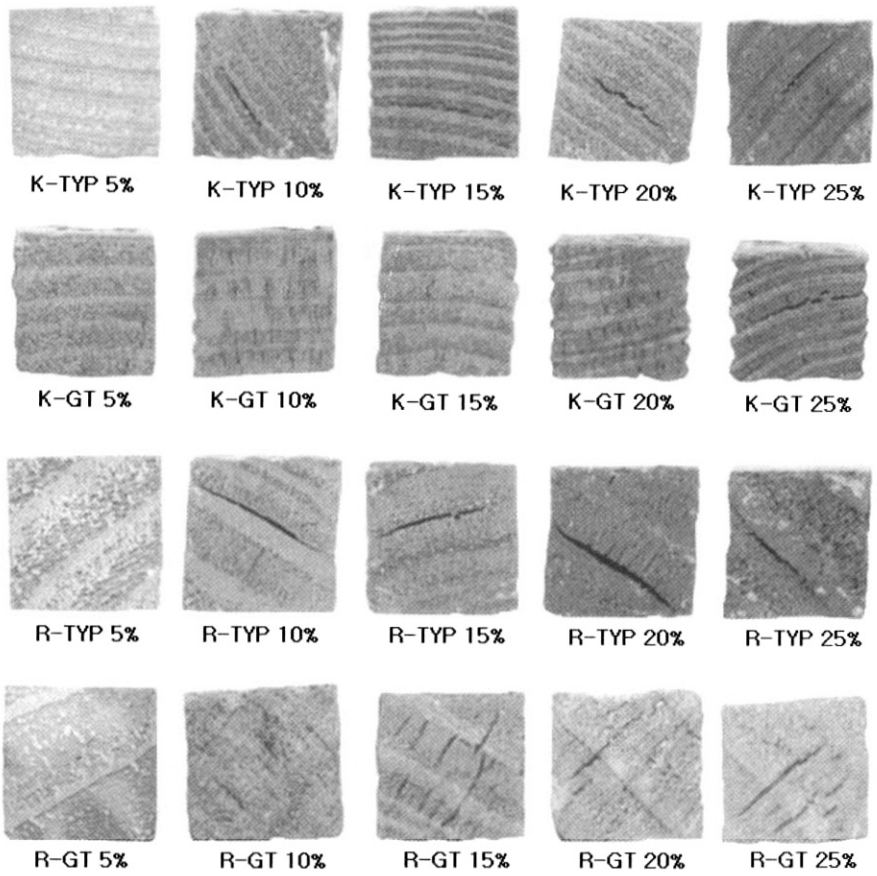

Fig. 1. Macroscopic features showing development of ring and radial checks in transverse surface. K: Korean red pine; R: radiata pine; TYP: *Tyromyces palustris*; GT: *Gloeophyllum trabeum*; %: Target weight loss level.

Paraffin was proved quite satisfactory embedding media in advanced stages of decay.

The method of embedding FAA-fixed specimens in polyethylene glycol, razor-blade cutting, and solvent-exchange drying (Wilcox 1964) was demonstrated to be an excellent technique in surface preparation of decayed wood for SEM studies. The mechanical support provided by polyethylene glycol matrix during razor-blade cutting and prevention of excessive shrinkage by the solvent-exchange drying could minimize artifacts during specimen preparation. By using this method, the turgid appearance and the natural position of fungal hyphae in decayed wood could be well preserved. Also, this method could keep microscopic integrity of decayed wood, thus facilitating the study on cell wall structure changes with the progress of decay. And ultrastructural changes by degradation of cell wall layers were correctly observed through transmission electron microscopy.

In the middle lamella, however, artificial defects of delamination and chatter marks occurred during sectioning. These defects were known to be caused by degradation of lignin (Eriksson *et al.* 1990). Some morphological features observed

in electron microscopy could be attributed to swelling and drying of cells during decay or specimen preparation (Liese 1970). Also, tracheids showing 'wavy' appearance were thought to occur during specimen preparation due to some loss of cell wall integrity (Blanchette *et al.* 1994).

3.3. Movement and Distribution of Hyphae

In the present study, most bore holes in the tracheid walls were thought to be produced during the early stages of decay because bore holes began to appear in the early stages of decay (Fig. 2B & C) and their number was relatively unchanged even in the advanced stages of decay. And hyphae penetrated mainly through bordered pits (Fig. 2A) but occasionally through the created bore holes (Fig. 2B). Thus, these bore holes were considered to be of help for hyphal penetration into adjacent tracheids from the early stages of decay.

Fungi moved from cell to cell through the natural cell wall openings of pits or the cell wall by creating bore holes. The formation of bore holes was considered the largest feature that distinguish brown-rot and white-rot fungi from other wood-inhabiting fungi. Wilcox (1968) insisted, however, that hyphae of brown-rot fungi extended from one cell to another mainly through pits in the early stages of decay but eventually penetrated through the cell wall by creating bore holes after significantly decayed. And Wilcox (1968), Liese (1970), and Messner and Stachelberger (1984) reported that bore holes were not frequently observed in early stages of brown-rot, and thus not to be a preference passage for movement of brown-rot fungi. Kwon (1993) noted that hyphae usually moved through cross-field pits after their deep penetration into ray cells because penetration of

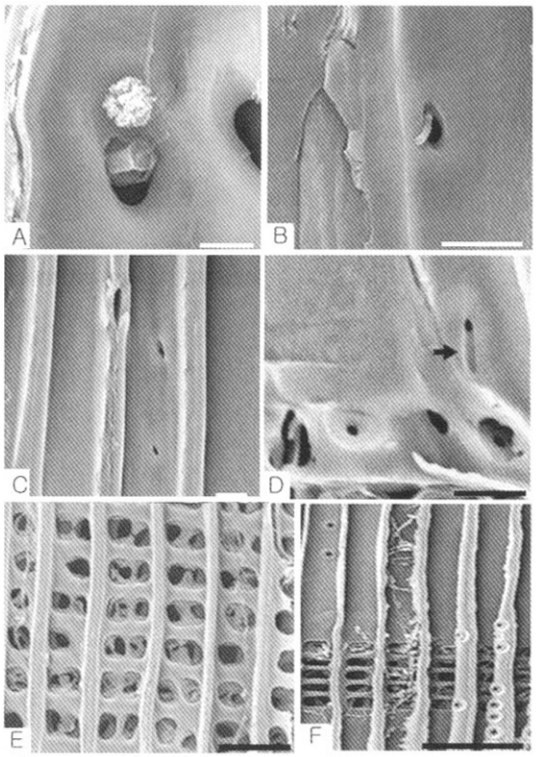

Fig. 2. Radial surfaces showing openings for passage of hyphae in tracheid walls (A~C) and penetration and distribution of hyphae (D~F). A (R-GT 10% weight loss): Penetration of a hypha through a bordered pit. B (K-GT 10% weight loss): Penetration of a hypha through a bore hole. C (K-GT 5% weight loss): Formation of slit-like bore holes lying at small angle to cell axis. D (K-GT 20% weight loss): Depressed hyphal trace (arrow). E (K-GT 5% weight loss): Hyphae passing through cross-field pits. F (K-TYP 20% weight loss): Hyphae-filled cross-field pits and a tracheid. A-F: SEM; Scale bars: A=5 μm, B-D=10 μm, E=50 μm, F=100 μm.

hyphae through bordered pits and bore holes were not found.

Hyphae normally bored not only circular holes passing through tracheid walls perpendicular to the cell axis but also slit-like holes lying at a small angle to the cell axis (Fig. 2C). Also, as decay progressed, depressed hyphal

trace was identified (Fig. 2D).

Liese and Schmid (1966) insisted that microhyphae observed in hyphal tip were related to physical penetration through wood cell wall and formation of bore holes. Highley *et al.* (1983b), however, suggested that microhyphae were not essentially required for degradation of cellulose because no penetration into the cell wall by microhyphae was revealed in 11 brown-rot fungi in electron microscopy. As the suggestion by Chou and Levi (1971) that bore hole created by hyphae was attributed to the source of enzymes causing the cell wall to degrade, microhyphae occasionally penetrated through the cell wall by creating bore holes although they penetrated primarily through bordered pits and cross-field pits (Fig. 2A & E). Therefore, microhyphae were thought to have more or less influence on the degradation of cell wall.

In the early stages of decay, though hyphae were observed more frequently in earlywood than in latewood, radial checks or cavities and degradation were found more extensively in latewood than in earlywood (Fig. 3A & B). Thus, the opinion of Waterman and Hansbrough (1957) that distribution of hyphae was not related with degradation proved to be true in the present study. In the advanced decay, hyphae were impartially distributed in both earlywood and latewood.

Most hyphae were also present commonly in the rays and penetrated into the adjoining tracheid lumina (Fig. 4C) and were distributed in lignin-rich cell corner regions in tracheid lumina (Fig. 3C). This fact might be correspondent with the description by Nilsson (1974) that wood components other than cellulose caused the cellulose to degrade.

Moreover, as decay progressed, hyphae with clamp connections and thick hyphae elongated along the tracheid axis were observed frequently and a number of the microhyphae were distributed throughout tracheid lumina. Hyphal colonization in cross-field pits was also observed commonly (Fig. 2F). In the advanced stages of decay, ray parenchyma cells, differently from ray tracheids, were mostly removed and filled with mycelia (Fig. 4D).

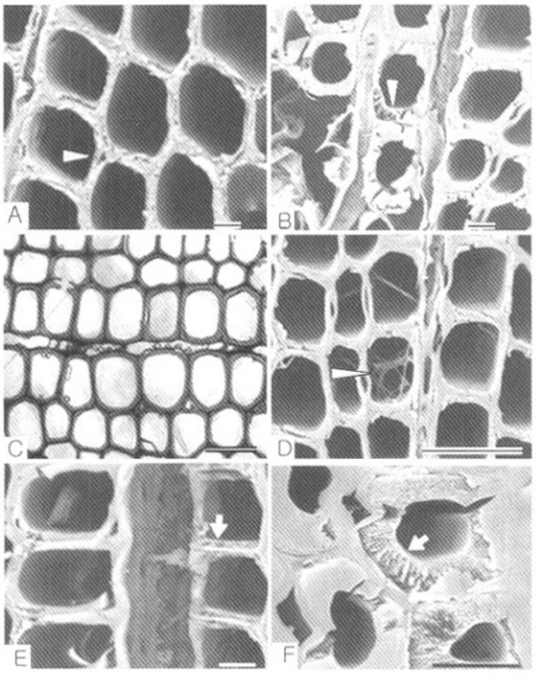

Fig. 3. Transverse surfaces showing structural changes of tracheid walls (A, B, E & F) and distribution of hyphae (C & D). A (R-TYP 5% weight loss), B (R-GT 5% weight loss) & F (K-TYP 15% weight loss): S_2 layer of the secondary wall having a porous appearance by development of radial checks or voids (arrowhead). C (R-TYP 10% weight loss): Hyphae located in ray and tracheids. D (K-GT 10% weight loss): Development of mycelia (arrowhead) in tracheid lumina adjacent to ray. E (R-TYP 15% weight loss): Extensively degraded S_2 layer but intact compound middle lamella and S_3 layer (arrow) in earlywood. A, B & D F: SEM; C: LM; Scale bars: A,B,E & F=10 μm. C & D=50 μm.

3.4. Ultrastructural Changes

In this study, hyphal sheaths that often encase hyphae of many brown-rot fungi during degradation of cellulosic materials were found even in the early stages of decay through TEM. Hyphal sheath was known to dissolve and absorb the cell wall decomposition products and to facilitate movement of enzyme through wood cell walls (Jutte and Sachs 1976; Leightley and Eaton 1980).

As decay progressed, most hyphae were considered not to participate in the direct degradation of the cell wall although they were located in the tracheid lumina. This fact might be attributed to the fact that diffusion of low molecular substances through fine apertures of wood cell walls caused the cell wall to degrade first. Also, it has been known that low molecular substances other than cellulases related to degradation of the cell wall were derived from cytoplasmic material of autolyzed hyphae (Highley *et al.* 1983a). In this study, however, no diffusion of cytoplasmic material of autolyzed hyphae was identified.

With the progress of decay, electron density in S_3 layer became higher than in S_1 and S_2 layers in the tracheid secondary wall. This fact was considered to be caused by osmiophilic particles like the reports of Messner and Stachelberger (1984) and Messner *et al.* (1986) that osmiophilic particles were reaction products of dihydroxyphenolic substances formed by demethylation of phenolic units and OsO_4. Therefore, in this study, the occurrence of partial demethylation of lignin was thought to occur in some tracheid walls (Fig. 4A).

Moreover, Messner and Stachelberger (1984), Highley *et al.* (1985), and Blanchette *et al.* (1994) reported that brown-rot fungi could degrade lignin because low electron density was identified in the middle lamella of cell corner. Kwon (1993) noted similarly that *Lentinus lepideus* could degrade lignin, based on the observation of decomposition of all cell wall components.

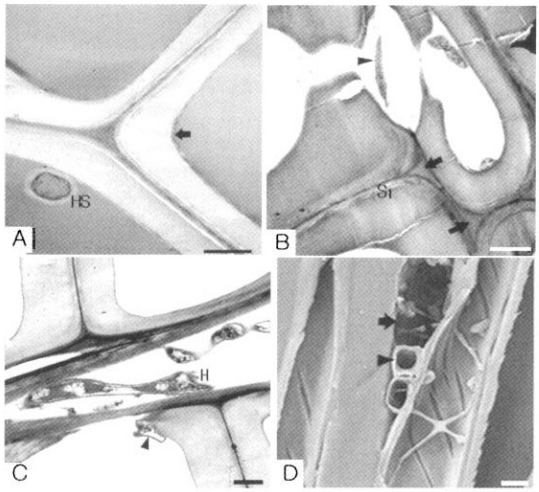

Fig. 4. Transverse surfaces showing ultrastructure of tracheid walls fixed with OsO_4 (A & B), distribution of hyphae (C), and tangential surface showing structural changes of ray cell walls (D). A (R-TYP 10% weight loss): Formation of hyphal sheath (HS) and osmiophilic (electron-dense) particles (arrow) in the S_3 layer of cell corner. B (K-TYP 25% weight loss): Revealed microfibrils by some decomposition of lignin in S_1 layer, low electron-dense true middle lamella in cell corner (arrows) but intact torus and margo (arrowhead). C (K-GT 10% weight loss): Hyphae (H) with sheath in ray and a hypha attached to innermost S_3 layer (arrowhead). D (R-GT 20% weight loss): More severely degraded ray parenchyma cell walls (arrow) than ray tracheid walls (arrowhead). A-C: TEM; D: SEM; Scale bars: A-C=2 μm, D=10 μm.

In the present study, low electron density in the middle lamella of cell corner region were found in the last stages of decay (Fig. 4B). Thus, brown-rot fungi used in this experiment was thought to produce hyphae causing decomposition of all cell wall layers including S_2 layer.

In this study, pit membrane, *i.e.* torus and margo, of bordered pits remained mostly intact

and compound middle lamella, *i.e.* primary walls of two opposing cells and the intervening true middle lamella, showed no trace of degradation except for occurrence of low electron density in the true middle lamella of cell corner region (Fig. 4B).

3.5. Decay Resistance of Each Cell Wall Layer

Relative decay resistance of different wood elements and cell wall layers against brown-rot fungi was known to be attributed to differences in cell wall density and chemical composition (Meier 1955) and to different degrees of lignification (Meier 1955; Wilcox 1968). Especially, lignin was considered to be the strongest component in degradation by microorganisms (Wilcox 1970; Kuo *et al.* 1988).

In this study, however, low electron density happened in lignin-rich true middle lamella in cell corner region in most tracheids. This was in agreement with Nilsson (1974) who reported that the presence of lignin in some way might be related to the production of cellulases since lignified jute fibers were easily degraded by brown-rot fungi but delignified fibers were not.

Extensive degradation of cell walls took place in ray parenchyma cells unlike ray tracheids (Fig. 4D), which might indicate ray parenchyma cells being the weakest in resistance to brown-rot fungi. In the early stages of decay, S_3 layer was not nearly degraded, but degradation and radial checks or voids in S_2 layer extensively happened (Fig. 3A & B). This might be attributed the fact that the porosity of the cell walls increased and linkages between cellulosic fibrils decreased with the progress of decay (Eriksson and Blanchette 1990). The occurrence of checks in the secondary walls was known to be the cause of excessive strength loss even at a low weight loss (Cowling 1961; Highley 1977).

As decay progressed, although hyphae were in direct contact with the S_3 layer, ultrastructural changes were most obvious in the S_2 layer without causing noticeable loss of the S_3 layer (Fig. 3F).

Brown-rot fungi were generally known to attack all cell wall layers at the same time (Cowling 1961; Wilcox 1968). And in the early stages of decay, S_3 layer was found to be hardly degraded unlike the extensive degradation in S_2 layer (Jurásek 1958, 1964; Highley *et al.*, 1985). Also, S_2 layer in the secondary wall of softwood tracheids was found to be the first layer removed by brown-rot fungi (Meier 1955; Wilcox 1970). This might be related to the lower lignin concentration in the S_2 layer than in the S_1 and S_3 layers in the secondary wall of softwood tracheids (Saka and Thomas 1982). And the greater brown-rot decay resistance of the S_1 and S_3 layers was thought to arise from a greater degree of lignification in these layers (Wilcox 1968).

Intact tracheid shapes in the advanced stages of decay and undegraded compound middle lamella even in the last stages of decay were confirmed in this study (Fig. 3E), as Wilcox (1968) reported that enzymes secreted from brown-rot fungi could be the cause of demethylation of lignin but not change lignin matrix in essential. Thus, compound middle lamella appeared the strongest layer in the resistance to brown-rot fungi.

4. CONCLUSIONS

The ultrastructural changes of cell walls attacked by the two brown-rot fungi were compared in this respect.

1) Hyphae usually passaged through bordered pits and cross-field pits but occasionally penetrated through the tracheid walls by creating bore holes. These bore holes were considered to

be apparently the passage for facilitating direct hyphal penetration into tracheids and to have more or less influence on degradation of cell walls.

2) Most hyphae were commonly observed in the rays and tracheid lumina adjacent to rays, especially in lignin-rich cell corner regions. Therefore, wood components other than cellulose, such as lignin, was thought to cause the cellulose to degrade.

3) With the progress of decay, electron density of S_3 layer became higher than S_1 and S_2 layers. Also, in the last stages of decay, low electron density in lignin-rich true middle lamella in cell corner regions appeared. This might indicate that the brown-rot fungi could be the cause of degradation of all cell wall components including demethylation of lignin.

4) Extensive degradation first occurred in ray parenchyma cell walls, differently from ray tracheid walls. Thus, ray parenchyma cells appeared to be the least resistant to brown-rot fungi.

5) Although hyphae were in direct contact with relatively lignin-rich S_3 layer, S_2 layer was extensively degraded and lignin-rich compound middle lamella remained intact. This might indicate that brown-rot decay was related to the degree of lignin and that compound middle lamella was the strongest layer in resistance of brown-rot.

6) Radiata pine macroscopically showed more ring and radial checks than Korean red pine.

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