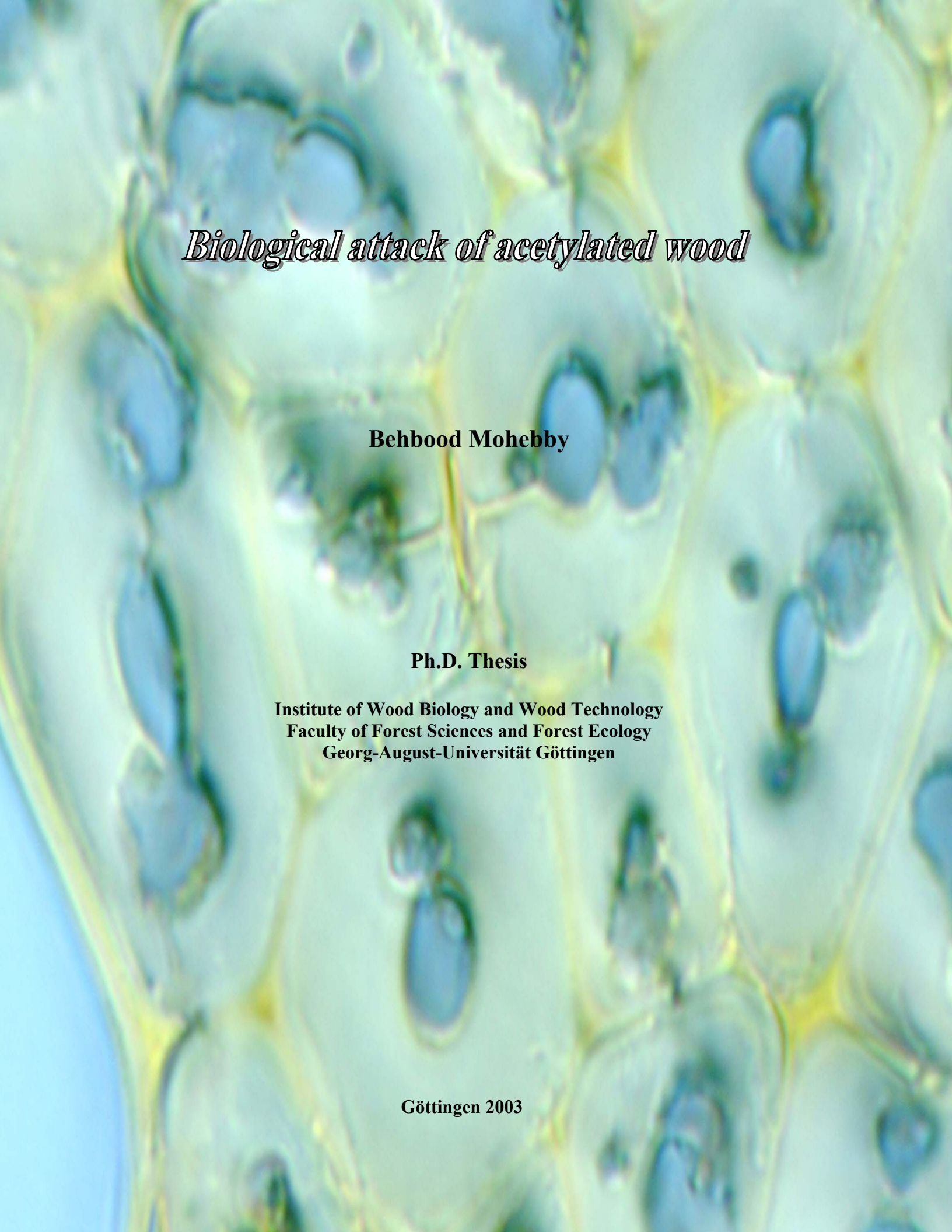

Biological attack of acetylated wood

Behbood Mohebby

Ph.D. Thesis

Institute of Wood Biology and Wood Technology
Faculty of Forest Sciences and Forest Ecology
Georg-August-Universität Göttingen

Göttingen 2003

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vorgelegt von

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To my dears who have spent their life for me:

Lobat, Yashar, Camellia

and great Parents

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Who does not thank God's creatures, does not appreciate him.

(Holy Koran)

Hereby, I would sincerely appreciate my merciful and great God who helped me all time in my life, in happy and sad moments.

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Abstract

Acetylation is an environmental friendly method to modify wood properties and protect it against biological attack. Many researches have been done in this field. However, no concern was paid for mode of protection in acetylated wood. This research had been aimed to study mode of action in acetylated wood by investigating on weight and MOE loss, chemical alteration in cell wall due to microbial attack, IR-spectroscopy, microcalorimetry, determination of fungal biomass by using ergosterol assay and FDA hydrolysis and enzyme assays. Beech and Scots pine wood samples were acetylated by using acetic anhydride at temperature 80-120°C for 180min. Acetylated wood samples were tested under soil bed test condition, filed and basidiomycete trials. Results showed that the losses of weight and MOE decreased at increasing weight gains. Soil bed test revealed that the acetylation of beech wood at above 8% and pine wood at above 10% inhibits soil microorganisms in attacking wood and their activities reached to nil at higher weight gains. A test with the white rot fungus (*T. versicolor*) showed that the weight loss decreased at raising weight gains. Weight gains of above 10% inhibited fungal decay in beech wood and it reached to zero at higher weight gains. Microscopical studies of wood from soil bed samples, field trials and basidiomycete (white- and brown-rot) tests showed that fungi could colonize acetylated and non-acetylated wood. However, measurements of fungal biomass by using ergosterol assays in those woods and fluorescein diacetate in white rot tests showed a rapid colonization of fungal hyphae at early stages of incubation and decreased amount of fungal biomass at raising weight gains. Results showed that fungal colonization is influenced by the acetylation. Biological activities were measured in wood by using microcalorimetry. The reduction of thermal powers and measured amounts of energy production in acetylated wood revealed that activities of microorganisms were influenced by the acetylation and their activities decreased at increased degree of the acetylation. Microscopy of field trial samples showed that the acetylation of wood was affected the growth of soil microorganisms and protected wood against soil microorganisms during a long period of exposure (350 weeks) to soil. Different types of decay in field samples showed successional activities of soft- and white-rot fungi and also bacteria. Soil bed test showed a synergism between soil fungi and bacteria in wood. It was revealed that bacteria followed hyphal traces in cells and associated with fungi in wood degradation. Chemical analyses of acetylated wood in soil bed samples showed a significant effect of the acetylation on removal of cell wall components. The analyses showed a reduction in removal of cell wall components at increased weight gains. Results revealed that removal of the cell wall components reduced considerably in beech wood at weight gains above 8% and in Scots pine samples at above 10%. Study on patterns and phenology of white- and brown-rot decay on acetylated wood showed no difference of decay patterns between acetylated and non-acetylated wood, however decay patterns appear more later in acetylated wood.

Keywords- acetylation, beech, Scots pine, mass and MOE_{dyn} loss, soil bed test, field test, basidiomycete, soft rot, bacterial degradation, white rot, brown rot, *Trametes versicolor*, *Poria placenta*, light microscopy, SEM, chemical analysis, IR spectroscopy, ergosterol assay, FDA analysis.

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Chapter 1

Research background

1.1. Introduction

Since last decade main concern was focused on the traditional wood preservatives that are presently used to improve durability of wood. Numbers of scientific research reports have been revealed that typical preservatives that contain toxic chemical bases; for example arsenic, zinc, copper, chromium or oil base chemicals; e.g. creosote, etc. are problematic for the environment. Reports indicate that creosote is carcinogenic (Karlehagen, 1990), arsenic leaches out of the treated wood and pollutes soils and waters (Bergholm, 1990; Garcia & Rowland, 2001; Solo-Gabriele *et al.*, 1998, 1999, 2000, 2002; Townsend *et al.*, 2001a,b&c, 2001; Hauserman, 2002a,b&c; DeWitt, 2002a&b; Gainesville Sun Newspaper, 2002a&b). Disposing the waste CCA treated wood in fire furnace also is not a right solution due to high ash content remaining after furnacing (Solo-Gabriele *et al.*, 2002; Cooper, 1990). Chromium is also toxic and produces dermal inhalation diseases (Chen *et al.*, 200). It leaches out due to rainfall (Solo-Gabriele *et al.*, 2002). Due to high pressure (public, media and law) against using toxic based preservatives, the use of these preservatives are being subjected to decrease because of their environmental impacts or some of them (for example CCA) are going to be banned in Europe (Germany and The Netherlands) and USA (Schert, 2002; EPA report, 2002) and Canada (PMRA report, 2002).

Considerable improvements have been made in formulation and fixation of traditional preservatives to prevent their leaching into environment, soil or water. However, there is still no real solution.

Wood modification is new approach to preserve wood from biological and climatological damages with environmental friendly chemicals. In wood modification the basic chemistry of cell wall polymers is altered which can change important properties of wood including durability, dimensional stability, hardness and UV-stability. Controlling the moisture content in wood is a very effective way to protect it from physical damages or some biological attack, especially fungal attack.

1.2. Chemical wood modification

Most of the researches in the field of chemical modification of wood were conducted for improving either its dimensional stability or its biological resistance (Matsuda, 1996). Wood is made up primarily of cellulose, hemicellulose and lignin. Originally, chemical modification of wood was a chemical reaction between some reactive parts of wood components and a simple chemical reagent to form a covalent bond between both wood and chemical (Rowell, 1975; Larsson, 1998). Hydroxyl groups in the wood polymers (i.e. cellulose, hemicellulose and lignin) are the most reactive sites in wood. They are also responsible for the dimensional instability through their hydrogen bonding with water. Chemical modification of wood by reaction of the hydroxyl groups in wood with a chemical reagent is substituting the hydroxyl groups with a stable, covalently bonded, less hydrophilic group, which leads to an increased dimensional stability (Larsson, 1998).

In order to chemical modification of wood, many chemicals capable of forming covalent bonds have been studied. The created bond between the wood polymers and the reagent is of great importance to make a permanent modification in wood. The major important types of covalent bonds formed by chemical modification of wood are ethers, esters and acetals (Matsuda, 1996; Larsson, 1998). Studies on chemical modification have been extensively reviewed over the last decades (e.g. Dreher *et al.*, 1964; Rowell, 1975; Rowell, 1982; Rowell *et al.*, 1994) and are more recently, this area has been reviewed by several authors (Kumar, 1994; Beckers & Militz, 1994; Militz & Beckers, 1994a; Beckers *et al.*, 1995; Matsuda, 1996; Militz *et al*., 1997; Beckers *et al.*, 1998; Rowell *et al.*, 1998; Larsson, 1998; Larsson, 1999 a&b; Gomez-Bueso *et al.*, 1999 a&b; Hill *et al.*, 2000; Chang *et al.*, 2000; Pan & Sano, 2000; Rosenqvist, 2001; Sander & Koch, 2001; Li *et al.*, 2001).

Chemical modification of wood improves its properties by altering the basic molecular structure of cell wall components. It implies the combination of two very different expertise, wood chemistry and wood anatomy. Thus, chemical modification of wood is very complex and requires a multidisciplinary approach. In many chemical modification of wood, reactions of hydroxyl groups play main leading role. In this case wood reacts as an alcohol. Many chemicals have been used to modify wood. The main reaction types are:

1.2.1. Etherification

Etherification of wood can be conducted by reacting wood with alkyl halides, acrylonitrile (AN), epoxides, β-propionolactone (acid conditions) and dimethyl sulfate (Matsuda, 1996). During the etherification the hydrogen within the hydroxyl group of a cell wall polymer is substituted by an alkyl group (fig. 1-1).

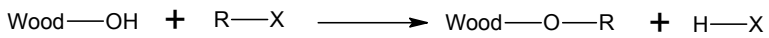

Fig. 1-1. Etherification of wood

The formation of an ether bond can be the result of an alkylation or epoxidation of the wood.

a. Alkylation

In the reaction of alkyl chlorides with wood, hydrochloric acid is formed as a by-product. Because of this, a great deal of wood degradation takes place during the reaction (Rowell & Banks, 1982). The simplest ether is formed during methylation of wood. This can be achieved by reaction with methylchloride (fig. 1-2).

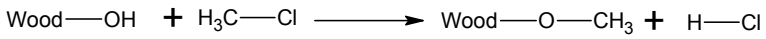

Fig. 1-2. Etherification of wood using methylchloride

Another methylation has been achieved by a treatment of wood with dimethyl sulfate or methyliodide (Militz *et al.*, 1997). Methylated wood though is mechanically impaired because of severe reaction conditions (Kumar, 1994) and the by-product formed. This alkylation of wood gives a high initial antishrink efficiency (ASE) but the effects of the alkylation are lost over time (Militz *et al.*, 1997). In case of reaction of wood with an alkyl chloride in pyridine, the ASE is not caused by the formation of an ether bond with holocellulose or lignin but by the formation of alkyl pyridnium chloride polymers which have the effect of bulking, but are easily leached out (Rowell & Banks, 1982).

b. Epoxidation

Another category of ether bond forming reactions is those between wood and alkylene oxides (fig. 1-3).

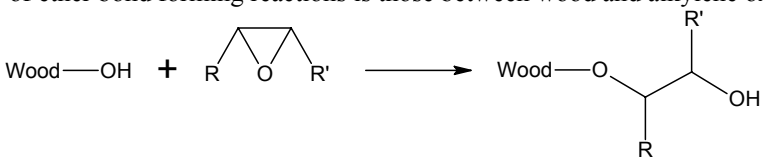

Fig. 1-3. Reaction of wood with an epoxide

The reaction of wood with epoxides is an example of a polymerizing addition. The used chemical reacts initially with a wood cell wall hydroxyl group and subsequently polymerizes by addition to the new formed hydroxyl group which arises from the epoxide. Several epoxides have been used the past decades for wood modification purposes. They include ethylene oxide (EO), propylene oxide (PO) and butylenes oxide (BO) (Norimoto *et al.*, 1992; Militz *et al.*, 1997; Rowell & Ellis, 1984; Akitsu *et al.*, 1993) and epichlorohydrin (EpCl) (Matsuda, 1993; Goethals & Stevens, 1994). The stability of the treated product and the effects generated vary with the reactant and method of treatment. Epoxidation takes place at elevated temperature and pressure. Usually the reaction is catalyzed under mildly basis conditions. In most experiments triethylamine (TEA) is used as a catalyst (figs. 1-4 & 1-5).

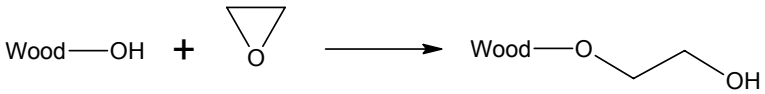

Fig. 1-4. Reaction of wood with ethylene oxide (EO)

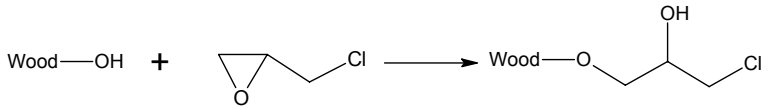

Fig. 1-5. Reaction of wood with epichlorohydrin (EpCl)

c. Benzylation

Benzylation has been carried out with wood meal to convert wood to thermoplastic materials. Different parameters were used for obtaining benzylated woods with different degrees of substitution. Results showed that pretreatment of the wood with NaOH as a swelling agent and water as a solving agent, as well as varying reaction temperatures, had critical effects on the benzylation reaction. The reaction proceeds by the following mechanism (Matsuda, 1996; Hiraoka *et al.*, 1997) (fig.1-6).

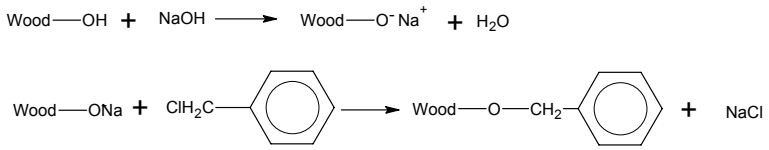

Fig. 1-6. Benzylation of wood with benzylchloride

d. Allylation

In this case, wood meal is pretreated with a NaOH aqueous solution and reacted with allyl chloride or allyl bromide (fig 1-7). It was found that allyl bromide gave better results than allyl chloride (Matsuda, 1996).

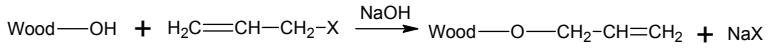

Fig. 1-7. Allylation of wood with an allyl halogen

e. Cyanoethylation

Reaction of wood with acrylonitrile (AN) produces cyanoethylated wood (Matsuda, 1996). In this case, before any reaction, wood is pretreated with NaOH aqueous solution and the degree of reaction is generally low (fig. 1-8).

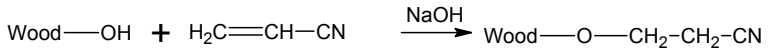

Fig. 1-8. Cyanoethylation of wood with ethylene cyanide

f. Acetals

A half acetal is formed by adding a carbonyl group of a modifying agent (aldehyde or kenton) to an alcohol (hydroxyl group of the wood cell wall polymers). This half acetal can further react with a second hydroxyl group of the cell wall polymers (cross-linking) and an acetal bond is formed (fig. 1-9). Several acids have been used as catalysts as well as sulfur dioxide (SO_2) (Akitsu *et. al.,* 1993).

One of the aldehydes, which is used and reported most frequently in literatures, is formaldehyde. This treatment was first reported by Tarkow and Stamm at 1953 (Matsuda, 1996). Akitsu *et. al.* (1993) used sulfurdioxide as catalyst. Yano & Minato (1993) and Yasuda & Minato (1994) treated wood samples using formaldehyde and SO_2 at 120°C for 24 hours (fig. 1-10).

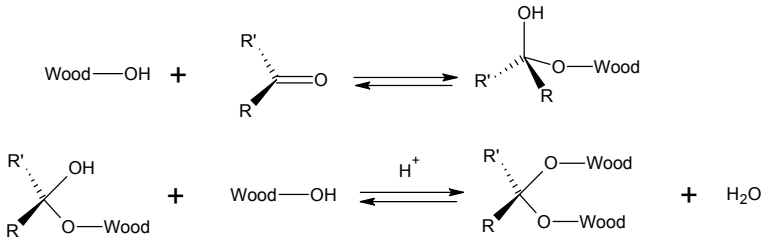

Fig. 1-9. Acetalation of wood

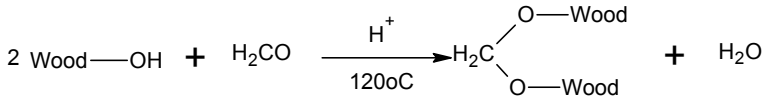

Fig. 1-10. Reaction of wood with formaldehyde

Besides formaldehyde other cross-linking chemicals have also been used. They include glyoxal, glutaraldehyde and dimethylol dihydroxyethylene urea (DMDHEU). Treatment with these reagents was carried out with SO_2, being an excellent catalyst for acetalation, by Yasuda & Minato (1994) and Yusuf *et. al.* (1994, 1995). Reactions were carried out at 120°C for 24 hours. The concentration used from 5 to 25% in water for glyoxal and glutaraldehyde. Other catalysts which have been used with DMDHEU are aluminum chloride, citric and tartaric acid. Treatments were carried out with aqueous solutions of 10-95% at temperatures of 80-175°C (Militz, 1993).

g. Aminals

As a special case of ether formation, the reaction between wood and N-hydroxymethylacrylamide (NHMA) could be mentioned (Goethals & Stevens, 1994) (fig. 1-11).

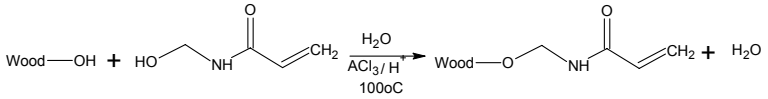

Fig. 1-11. Reaction of wood with N-hydroxymethylacrylamide (NHMA)

1.2.2. Esterfication

Esters are formed by reaction of wood with carboxylic acids or acid anhydrides (fig. 1-12).. Ester bonds are liable to acid or base attack, which leads to hydrolysis.

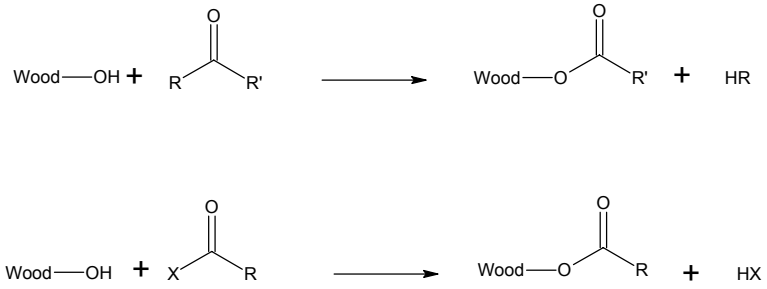

Fig. 1-12. Esterification of wood; R: Alkyl group or proton (H) , X: Halogen

a. Carboxylic acids

During the reaction between wood and carboxylic acids water is produced as a by-product (fig. 1-13).

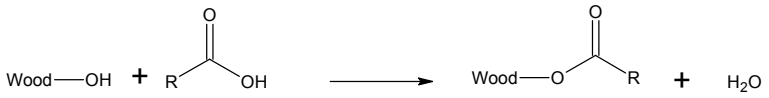

Fig. 1-13. Reaction of wood with carboxylic acid

Acetylation with acetic acid as well as acetylation with trifluoroacetic acid, halogen-substituted carboxylic acids and malonic acid, succinic acid, glutaric acid, adipic acid and sebatic acid has been tried. Beech sawdust has been treated with these latter ones using benzene as a solvent for a reaction of 18-24 hours at room temperature (Militz *et al.*, 1997).

b. Anhydrides

During the esterification of wood with anhydrides the acid within the anhydride is produced as the by-product if an alkylic anhydride is used. A new hydroxyl group is formed when esterification is performed with a cyclic anhydride. This new hydroxyl group can cross-link with another hydroxyl group of cell wall polymers in a possible acetalation reaction (fig. 1-14).

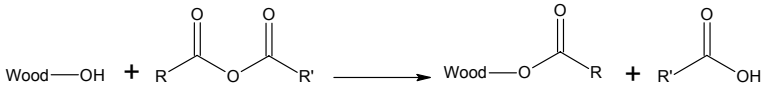

Fig. 1-14. Esterification of wood using alkylic anhydride

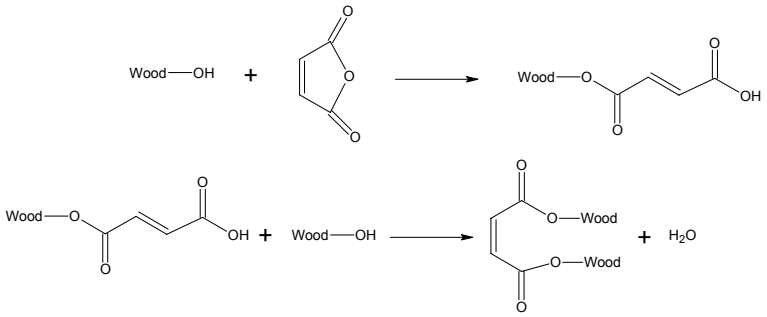

Fig. 1-15. Reaction of wood with maleic anhydride including a succeeding acetalation reaction

An advantage of acetylation with anhydrides is that for most of them no catalyst is required. Several anhydrides have been examined such as propionic and butyric anhydride (Goldstein *et al.*, 1961; Stamm & Tarkow, 1947; Hill & Jones, 1996), phthalic anhydride (Popper and Bariska, 1975), maleic anhydride (Matsuda, 1992), glutaric and 1,2-cyclohexanedicarboxylic anhydride (Goethals & Stevens, 1994). Most studies though of all chemical modification treatments for wood have been acetylation using acetic anhydride. During the reaction of the wood with the anhydride, hydroxyl groups of holocellulose and lignin are substituted by less hydrophilic acetyl groups. Acetic acid is produced as by-product. Dozens of authors have reported their results on acetylation of fibers, chips, veneers and solid wood. Several catalysts and solvents have been used such as xylene (Goldstein *et al.*, 1961), trifluoroacetic acid (Arni *et al.*, 1961), dimethylformamide and urea-ammonium sulfate (Clermont & Bender, 1957). Like many other anhydrides, esterification with acetic anhydride proceeds well, even in the absence of catalysts (Rowell *et al.*, 1986).

c. Dicarboxylic acid anhydrides

When dicarboxylic anhydrides are reacted with wood, esterified wood bearing carboxyl groups are obtained (Matsuda, 1996). Reaction is shown below (fig. 1-16).

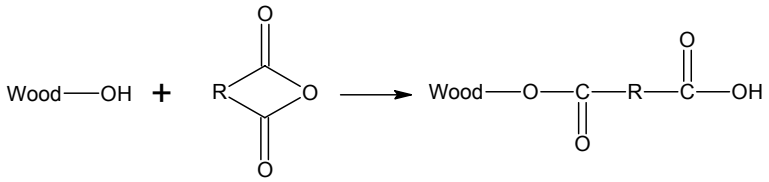

Fig. 1-16. Reaction of wood with a dicarboxylic anhydride

The early work was done with phthalic anhydride (PA) (Matsuda, 1996). Efficient reaction producing a series of carboxyl group-bearing esterified wood obtained by addition reaction of wood meal with maleic anhydride (MA), phthalic anhydride (PA) and succinic anhydride (SA). The reaction proceeds at room temperature in *N,N*-dimethylformamid (DMF) and dimethylsulfoxide (DMSO).

d. Oligoesterification

The carboxyl groups introduced into wood by above reaction (fig 1-16) are also reactive with epoxy groups. Epoxide-adducted esterified woods were obtained by reaction of the carboxyl group-bearing esterified wood meal with epoxides as shown in fig. 1-17 (Matsuda, 1996).

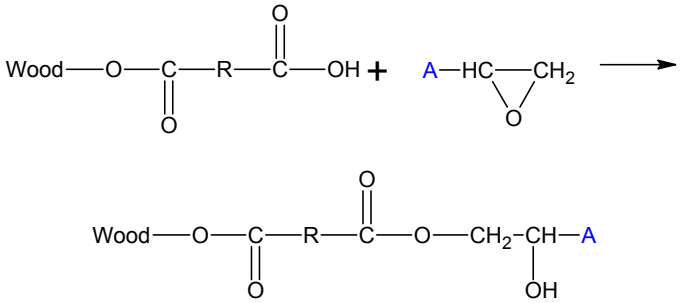

Fig. 1-17. Epoxide-adducted esterified wood produced by a reaction between carboxyl group-bearing esterified wood with an epoxide

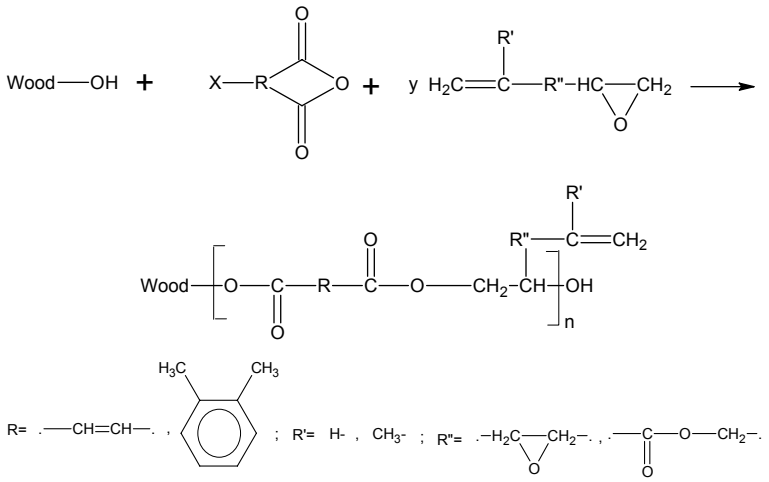

Fig. 1-18. Production of oligoester

In this case, phenylglycidyl ether (PGE), allylglycidyl ether (AGE) and glycidyl methacrylate (GMA) were used as the epoxides. When the epoxide-adducted esterified wood meals were further allowed to react

with the anhydride and the epoxide at high temperatures, alternatively adding esterification reactions occurred, to produce oligoesterified wood. Polymerizable oligoester chains have been introduced into wood meal with the anhydrides and the epoxides such as AGE or GMA as follows (fig 1-18) (Matsuda, 1996).

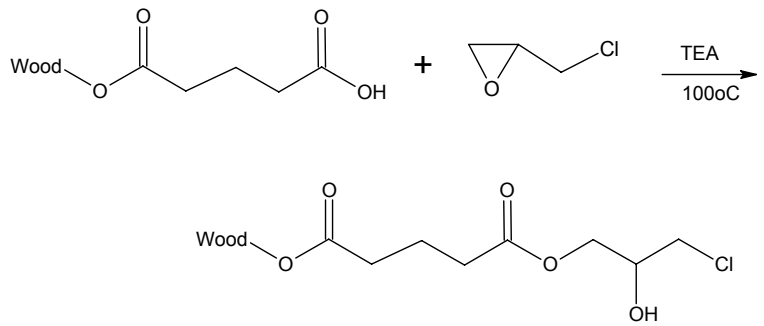

Fig. 1-19. Epoxidation of wood with epichlorohyrdin treated with anhydride

Reaction of some anhydrates with wood yields unstable ester bonds or create a new hydroxyl group when cyclic anhydrides are used. These later ones have the advantage that no by-product is formed. An additional epoxidation reaction eliminates the hyrdphylic carboxyl groups produced by the esterification. This oligoesterified wood has successfully been produced using phthalic or maleic anhyrdride in combination with epichlorohydrin (Matsuda, 1993) and glutaric anhydrides with epichlorohydrin (Goethals & Stevens, 1994). These oligoesterifications were carried out in a solution of dimethylformamide at 100°C for 4 hours using triethylamine (TEA) as a catalyst (fig. 1-19 & 1-20). Samples can be treated in a one step or two steps reaction.

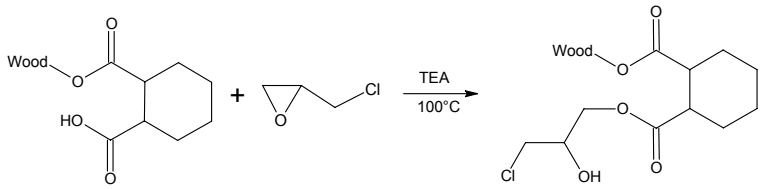

Fig. 1-20. Epoxidation of wood with epichlorohydrin which had been esterified with 1,2-cyclohexane dicarboxylic anhydride (Goethals *et al.*, 1996)

1.2.3. Urethanes

Another class of reactive chemicals, which has been studied extensively, is the isocyanates. In the reaction of wood hydroxyl groups with isocyanate a urethane bond (nitrogen-containing ester) is formed (fig. 1-21). Unlike mono-isocyanates, a reaction of wood with di- and poly-isocyanates can result in polymerization or self-polymerization/bulking.

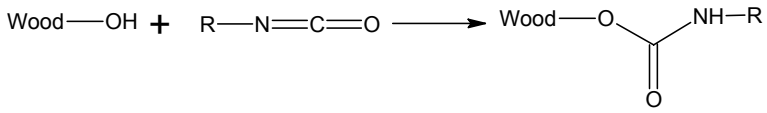

Fig. 1-21. Reaction of wood with a mono-isocyanate

Methyl-, ethyl-, n-propyl-, n-butyl-, phenyl-*p*-tolyl-, isocyanate and 1,6-diisocyanate hexane and tolene-2,4-diisocyantes all have been used to modify wood cell wall polymers (Militz *et al.*, 1997). A frequently used isocyanate in fiber technology is 4,4-diphenylmethane diisocyanate (MDI). Isocyanates swell wood and react with it at 100°C to 120 ^{o}C without a catalyst or with a mild alkaline catalyst such as triethylamine (TEA). The resulting urethane bond is very stable to acid and base hydrolysis. There are no by-products generated from the chemical reaction of isocyanate with dry wood.

1.2.4. Oxidation

Chen & Rowell (1989) and Goethals & Stevens (1994) used sodium periodate and periodic acid for oxidation of solid wood. Wood treatment with aqueous solutions (1-3%) of these chemicals at 25 °C for 24 hours resulted in limited oxidation of the cell wall polymers (fig. 1-22). This was shown by infrared spectroscopy in both research experiments.

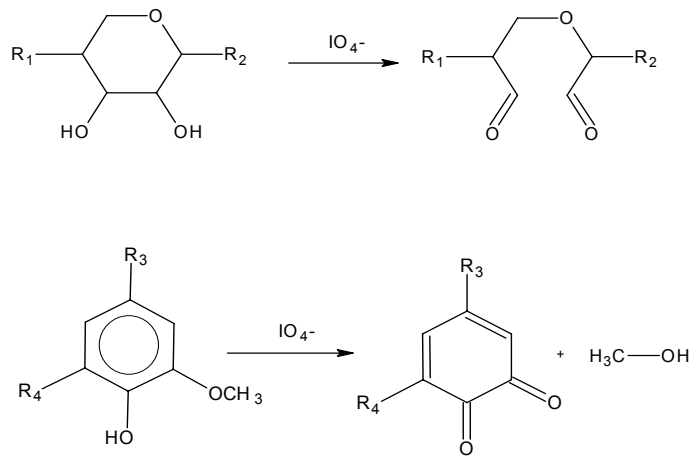

Fig. 1-22. Oxidation of wood cell wall; Top: Holocellulose; Bottom: Lignin; R_1 and R_2: Holocellulose unit; R_3: Lignin side chain; R_4: H, OCH_3 or lignin unit

1.2.5. Silylation

The natural durability of silicate containing wood species such as *Dicorynia guyanensis* (Angelique) has led to experiments with chemical reactions of wood with organic silicon compounds such as chlorosilanes and alkoxysilanes.

Among the variety of treatments described various chemicals and formulations based on silicon compounds have been used. These treatments can be divided into the following systems [1]:

- Inorganic silicates
- Sol-gel mechanism
- Micro-emulsion technology

a. Inorganic silicates

Chlorosilane treatment of various wood species was firstly reported by Owens *et al.* (1980) Wood blocks (20 x 20 x 10 mm) of red pine sapwood, Douglas fir sapwood and Douglas fir heartwood were treated by immersing them for 60 seconds in $SiCl_4$ at room temperature and then cured at 104°C /fig. 1-23).

Chlorosilanes react very fast with wood components but the main disadvantages of this reaction are the formation of hydrochloric acid as a by-product, causing wood degradation, and the chemicals themselves being very moisture sensitive (Stevens, 1985). Propyltrimethoxysilane has been used by Goethals & Stevens (1994) at concentration of 1-25%. Curing was done at 100°C for 48 hours. Rubberwood has been treated with γ-methylacryloxypropyltrimethoxysilane by Rozman and coworkers who used a 50% solution in methanol and curing for 5 hours at 110-115°C (Militz *et al.*; 1997).

Water glasses are potassium or sodium silicates or solutions thereof. A typical water glass is composed of 2 – 4 mol silicate and 1 mol alkali oxide (Römpp,1995).

[1] Detailed information is given by Mai & Militz (2002).

Wood treatment with water glasses has been most extensively studied by Furuno's group (Furuno,1992). They have treated different veneers of hinoki (*Chamaecyparis obtuse*), kaba (*Betula maximowicziana*) and buna (*Fagus crenala*) in a two step process (Furuno *et al.*,1991). In the first step, the wood samples were impregnated with sodium water glass solutions ($Na_2O{\cdot}nSiO_2$, n = 2.06-2.31) in concentrations between 5 and 80% using either vacuum-impregnation or diffusion penetration at atmospheric pressure (little differences in the up-take were observed related to the impregnation technique). In the second step, the specimens were infiltrated with metals salt solutions such as aluminium sulphate or calcium chloride in order to precipitate the silicate within the wood structure by replacing sodium ions in water glass. The wood specimens were then dried at 60°C for 24h and finally under vacuum on phosphorous pentoxide for 24 h.

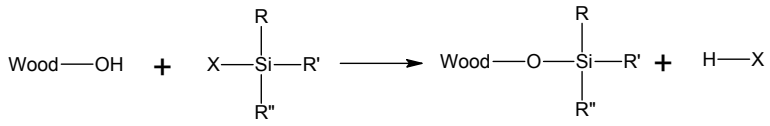

Fig. 1-23. Silylation of wood with an alkylsilane

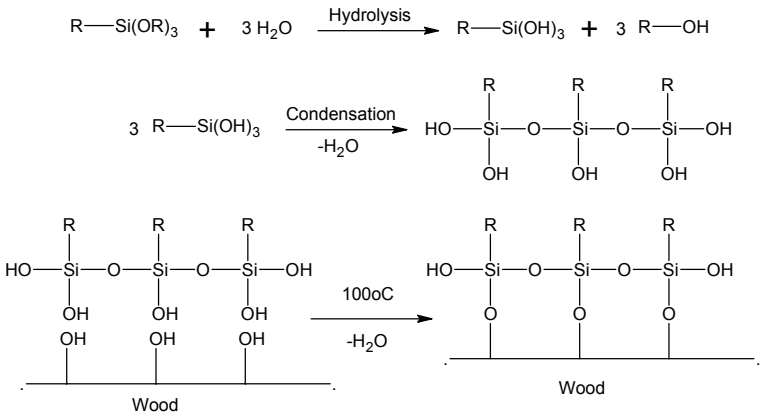

Fig. 1-24. Reaction of wood with an alkoxysilane (according to Goethals *et al.*, 1996)

b. Sol-gel mechanism

Wood modification applying the sol-gel process of silicon alkoxides has been reported by several research groups. The application of the sol-gel process was most intensively studied by Saka et al. (2001 a&b). Their impregnation technique was aimed to use the bound water in the cell wall in order to direct the sol-gel process to the cell wall and to achieve a deposition of the silicate therein (Saka *et al.*, 1992). A commercial application of gels based TEOS for the treatment of wood is described in the patent literature (Böttcher *et al.*, 2000).

A variation of the sol-gel process, which applies tetraalkoxy silanes and produces inorganic glasses consisting of pure polymeric SiO_2 is the use of organo-silanes (fig. 1-24). These are bifunctional molecules which contain three silicon-functional alkoxy groups, mainly methoxy and ethoxy groups, and an organo-functional group, which increases e.g. the hydrophobicity of the gel or forms a covalent bond with the cell wall polymers. Organo-silicons have a great variety of applications such as adhesion promoters, surface modifiers, cross-linking agents etc. The application of organo-functional silanes was mainly studied by Saka's group (Miyafuji & Saka, 2001; Saka & Tanno, 1996; Saka & Yakake, 1993).

c. Micro-emulsion technology

Coatings and primers based on the micro-emulsion technology have been developed for surface treatment of wood and masonry. The system consists of different silicon polymers in form of so called micro-emulsion in water with a particle size from 10 to 80 µm (Gerhardinger *et al.*, 1996; Hager, 1995). In

comparison to "macro"-emulsions of oil phase in water which require an emulsifier the micro-emulsion technology applies an additional co-emulsifier that interferes with the quasi-crystalline monomolecular surfactant film. In doing so a particle size of a few ten nanometres is obtained while that of "macro"-emulsions amounts to 1000 µm and more. Because of their minor size the micro-emulsions are able to penetrate into the voids of wood which cannot be reached by conventional emulsions. The micro-emulsion typically consists of an agent to be emulsified (silane, siloxane or polysiloxane), an emulsifier (silane, siloxane) and a co-emulsifier (functional polysiloxane). Both emulsifier and co-emulsifier in the micro-emulsion technology are active ingredients at the same time and lose their ability to emulsify after drying. When poured into water the micro-emulsions are activated since hydrolysis and condensation occurs. Therefore, dilution should take place directly before the application due to a growing particle size. The application of SMK micro-emulsions on wood caused high water repellency (reduction of water uptake up to 70% after two years of natural exposure) and prevented micro-cracks (fiber separation) due to weathering (Hager, 1995).

1.3. Acetylation of wood

Chemical modification of wood by acetylation with acetic anhydride is considered worldwide to be one of the most promising ways of enhancing wood properties taking into account both technical and economic aspects.

Acetylation involves a chemical reaction between hydroxyl groups in wood polymers, mainly the hydroxyl groups in lignin, hemicellulose and amorphous parts of cellulose. The reaction results in the formation of covalently bonded groups in wood and formation of acetic acid as a byproduct (fig. 1-25). Acetic anhydride also reacts with water forming acetic acid (fig. 1-26).

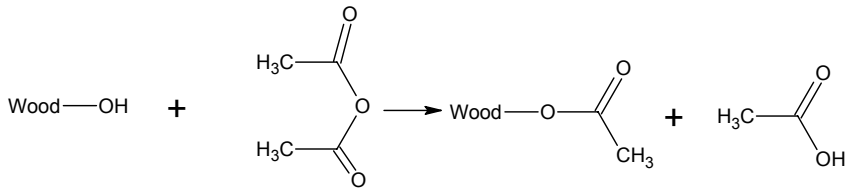

Figure 1-25. Acetylation of wood with acetic anhydride

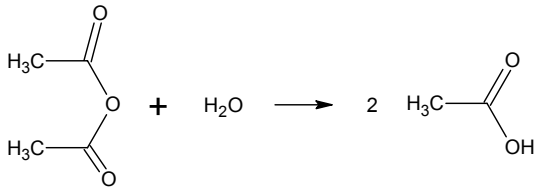

Figure 1-26. Reaction of acetic anhydride with water

1.3.1. Acetylation processes

The earliest studies on the acetylation of solid wood were done by Stamm *et al.* (1947), Tarkow *et al.* (1950) and Goldstein (1961). The reaction with acetic anhydride can be conducted in a ***liquid*** or ***vapor*** phase. They acetylated 3mm thickness veneers with acetic anhydride in liquid and vapor phases using pyridine helps open up the cell wall structure and act as a ***catalyst***. The liquid phase acetylation was performed under reflux condition, while in the vapor phase treatment the wood veneers were placed in the saturated vapor phase above the acetylation mixture. Acetylation at 90°C gave in both cases, acetyl content of 21% and the reaction time varied between 6 to 12 hours, depending on the wood species treated. The uptake of acetic anhydride was about 24 times as high for liquid phase treatment as vapor phase treatment (Larsson, 1998).

Most chemical modifications of wood are done with the addition of catalyst. The requirement of such catalyst completely depends on the used chemical and chemical bond aimed for. Many catalysts have used in acetylation procedures, such as zinc chloride, dimethyl formamide, magnesium perchlorate and sodium acetate reviewed by Rowell (1975). The vapor phase reaction could be carried out without a catalyst to

attain weight gain 20% in 2 hours at 130 ^{o}C with white pine cross section (3 mm in thickness). Addition of 15% dimethyl formamide (DMF) gave an acetyl content of 25% under the same condition.

Goldstein *et al.* (1961) acetylated wood without a catalyst. They used a mixture of acetic anhydride and xylene without any catalyst at 100-130°C under vacuum/pressure (Matsuda, 1996; Larsson, 1998). It was found that the use of extensive amount of acetic anhydride could be avoided by adding just the needed amount to the solvent. Byproduct acetic acid and excess acetic anhydride were removed from the wood by vapor drying with the solvent, followed by evaporation in a final vacuum step. This process was carried out in a pilot plant. But it was abandoned due to high recovery costs for the solvent.

Other chemicals also have been used instead of acetic anhydride, e.g. gaseous keten by Karlson and Shvalbe (ref. Larsson, 1998). The ketene gas was produced by pyrolysis of diketen and reacted it with wood at 55-60°C for 6 to 8 hours. The obtained weight gain was 19-22% and there was no byproduct. But it was avoided to use due to difficulties in its handling and toxicity, instability and lower dimensional improvement (Larsson, 1998).

A simplified procedure for acetylation is described by Rowell *et al.* (1985, 1986a&b). The wood particles were impregnated by dipping in acetic anhydride for a short period of time and then drained before going to a preheated reactor. After the completed reaction, unconsummated acetic anhydride and formed acetic acid are removed by means of evacuation. The removed anhydride can then be concentrated and recirculated to the impregnation. A research by Nilsson *et al.* (1988) on fungal resistance of particle boards made of acetylated chips in various procedures showed that acetylation with a limited amount of liquid acetic anhydride without any catalyst or organic co-solvent was preferred. According to the simplified procedure, Rowell *et al.* (1990) suggested that the impregnation solution in which the wood particles are dipped can contain up to 30% acetic acid, with a maximum reaction rate at a concentration of 10-20% of acetic acid. In this procedure the uptake of acetic anhydride can be decreased from the 130-135% obtained after dipping and draining to about 60% for oven dried wood without any negative effects.

The newest process of acetylation, which is patented by Militz & Beckers (1994b), is described as following steps: (***a***) providing the solid wood in a treatment container, (***b***) impregnating the material with cold or warm acetic anhydride as acetylating medium, (***c***) leaving the impregnated wood in the acetic anhydride for further reaction, (***d***) draining the solid wood from excessive acetic anhydride, (***e***) applying a vacuum to remove non-reacted acetic anhydride and formed acetic acid, and (***f***) post-treating the acetylated solid wood with steam or water to remove remaining acetic anhydride and the byproduct acetic acid.

The newest research conducted by Hill *et al.* (2000) showed a use of the hypernucleophile 4-dimethylamino pyridine was found to be the most effective catalyst at a concentration of only 1% of the catalyst in acetic anhydride. The weight percent gain of 20% was realized compared with 7% for the uncatalysed reaction after 30 minutes system at 100°C. Larsson *et al.* (1999) suggested using microwave heating during the treatment instead of catalyst.

1.3.2. Properties of the acetylated wood

Influence of the acetylation on wood properties depends on the method that is used. Uptake of acetic anhydride, reaction time, reaction temperature, initial moisture content, possible residual acetic acid in the wood and catalyst type and amount will influence chemical, physical and mechanical properties in acetylated wood. The effects of acetylation on wood properties are discussed below.

a. Dimensional stability

It is now very well known that the acetylation improves dimensional stability in solid wood and other lignocellulosic composites. Hydrophobicity and antishrink efficiency (ASE) of wood caused by the acetylation is due to chemical blocking of hydroxyl groups.

Tarkow *et al.* (1950) reported an improvement in dimensional stability of about 70% for maple and balsa wood acetylated to a WPG 20% and Spruce acetylated wood to WPG 26% (Larsson, 1998).

Popper and Bariska (1975) reported a 75% of improvement in dimensional stability of fir wood due to the acetylation after 8 hours of soaking/drying procedure. They believed that the dimensional stability of acetylated wood could be attributed to the chemical blocking of the polar alcoholic and phenolic hydroxyl groups of the wood.

Acetylated flakeboards modified with a mixture of acetic anhydride and xylene (50/50) showed $^1/_6$-$^1/_7$ of thickness swelling of control boards after either immersion in water for periods of up to 10 days or exposure to 90% relative humidity for periods of up to 20 days (Youngquist *et al.*, 1986).

Rowell and Rowell (1989) acetylated Scandinavian Spruce chips and reported a maximum reduction in equilibrium moisture content (EMC) is achieved at about WPG 20%. Rowell *et al.* (1991) also reported a reduction about 50% in EMC of acetylated fiberboards compared with control at same relative humidities.

Feist *et al.* (1991) modified aspen wood with acetic anhydride, methacrylate and a mixture of both to compare moisture sorption with untreated wood. They reported that the rate of moisture sorption of aspen acetylated to WPG 18% was greatly reduced in liquid water. But methacrylated wood reduced slightly the rate of swelling in liquid water.

Rowell *et al.* (1993) tested acetylated Southern Yellow Pine wood under various pH, temperature and moisture conditions and reported that at 24°C, acetylated wood was more stable at pH 6 than pH 2, 4 or 8. At 50°C and 75°C, acetylated wood was more stable at pH 4 than other pH values.

Hill and Jones (1995) performed a water soak/oven-dry cycling tests on modified Corsican pine wood with acetic, propionic and butyric anhydrides at a variety of WPGs. The results showed a higher dimensional stability in acetylated wood compared to others.

Ramsden *et al.* (1995) modified Scots pine wood chips with a mixture of acetic anhydride and xylene. They reported that the acetylation improved significantly the hydrophobicity and dimensional stability of the wood. An ASE of about 80% is achieved by due to the acetylation (Beckers & Militz, 1994; Militz *et al.*, 1997).

Acetylated bagasse fiber and fiberboards made from acetylated fiber at WPG about 17% had an EMC of about $^1/_3$ that of controls at all relative humidities tested. Acetylation with WPGs <24% reduced EMC in wood of about >30% in changing relative humidity between 30-80% and 25°C (Yano *et al.*, 1993).

Fiberboards made from acetylated fiber were more dimensionally stable than the boards made from steam-treated fiber at all specific gravity levels tested (Rowell *et al*., 1995).

Chow *et al.* (1996) reported a reduction of water sorption and thickness swelling in hardboards made from acetylated fibers. Also linear expansion in the dimensional stability test (from 30% to 90% relative humidity) was significantly reduced by the acetylation. Rowell (1996) reported a maximum reduction in EMC is achieved at about 20% due to the acetylation.

Gomez-Bueso *et al.* (1999 a&b) reported that the acetylation of fiber in composite products made from lignocellulosic fibers with different sources showed that regardless of the source of lignocellulosic materials, acetylation has a very positive impact on the performance of fiber composite products. For example, the thickness swelling for softwood fiberboard in water reduced by approximately 90%.

Acetylated flakeboards produced from *Gmelina arborea* and *Picea sitchensis* were much more dimensionally stable than untreated boards (Fuwape & Oyagade, 2000).

b. Biological resistance

The effectiveness of chemical modification in enhancing biological resistance has been assumed to be mostly due to cross-linking, bulking, or a combination of both for dimensional stabilization. Hydroxyl groups in cell wall polymers are not only the water adsorption sites but also the biological enzymatic reaction sites. Wood rotting fungi and termites have a very specific enzyme system capable of degrading wood polymers into digestible units. Therefore, if the substance for these systems is chemically changed, this enzymatic action cannot take place (Takahashi, 1996).

b.1. Soft rot decay and Bacterial decay

Nilsson *et al.* (1988) tested acetylated particleboards in soil and different fungal cellars and reported that at a WPG of about 15%, no microorganisms' attack was observed after 12 month.

Rowell *et al.* (1989) made acetylated veneer-faced particleboards and tested them in standard soil by exposing to two fungi, *Tyromyces palustris* and *Trametes versicolor*. Results showed an excellent fungal

resistance to both fungi in an 8-week soil block test. Also during 150 days bending creep test, the totally acetylated boards showed no strength or weight loss during exposure to *T. palustris*.

A study on lignin biodegradation with *Phanerochaete chrysosporium* showed less alteration of lignin occurs after acetylation of lignin DHP (dehydropolymers of coniferyl alcohol) (Kern *et al.*, 1989).

Beckers and Militz (1994) reported that a WPG of 10% was sufficient to prevent a soft rot attack on acetylated beech, pine and poplar, while a WPG of more than 20% was required to prevent acetylated pine from attack by brown rot fungi.

Larsson *et al.* (1997) tested acetylated wood in ground contact and reported that acetylation has a major impact on fungal resistance of wood. The resistance of acetylated wood at WPG of about 20% is in the same range as that of wood with higher retention of preservatives (copper-chromium based).

Larsson *et al.* (2000) reported that exposure of mini-stakes to three different unsterile soils in the laboratory showed that decay was significantly reduced at acetyl content of 15.1%. An acetyl content of 18.5% prevented most attack by brown, white rot and soft rot fungi. Acetyl levels above 20.9% were required to eliminate attack by tunneling bacteria. Also results showed that acetylation of wood gives only a minor protection against marine borers, although the degree of attack is lowered by increased acetyl content.

b.2. White and brown rot decay

Goldstein *et al.* (1961) determined the resistance of acetylated Southern yellow pine to wood destroying fungi in a 3-month laboratory test and concluded that a 17% of WPG was sufficient to void attack.

Peterson and Thomas (1978) tested white and brown rot fungi and reported that the acetylation of wood has a high effect on decay at WPG of about 15% or more.

Rowell *et al.* (1988) compared aspen flakeboards made from acetylated (WPG 18%) and non-acetylated flakes bonded with isocyanate and phenol formaldehyde adhesives subjected to bending creep test under progressive brown rot fungal attack with *Tyromyces palustris* and measured deflection of the boards. Results showed lesser loss of deflection in acetylated boards due to decay after 100 days. Also it was indicated that the weight loss was 1% and 0% for isocyanate and phenol formaldehyde bonded acetylated boards respectively.

Takahashi *et al.* (1989) reported that enhancement of decay resistance by acetylation varies with used fungi and wood species. For example, for a brown rot fungus (*Tyromyces palustris*), the recorded weight loss was nil at WPG 20% and for a white rot fungus (*Coriolus versicolor*) is also the same at WPGs 12-15% at perishable hardwoods.

Militz (1991) tested durability of acetylated beech wood against three types of brown rot fungi and reported no decay in treated wood.

Acetylation of pine sapwood to a WPG of 10.7% already prevented fungal attack. For poplar a WPG of 14.4% and for beech a WPG of 12.8% was required to achieve the same (Beckers *et al.*, 1995).

Takahashi (1996) revealed that brown rot fungi were more resistant to acetylation than white rot and soft rot fungi. About 20% of WPG is required to eliminate decay by *Tyromyces palustris* (brown rot) in any wood species. On the other hand, *Coriolus versicolor* (white rot) failed to attack acetylated Japanese cedar even at 6% of WPG, although 20% WPG was necessary to suppress its decay in Japanese beech and albizzia (tropical fast growing hardwood). The well known brown rot fungus *Serpula lacryman* was also less prevented by acetylation than *C. versicolor*. *T. palustris* was still the most aggressive fungi between brown and white rot fungi, based on the results of a soil burial test with acetylated wood.

Assessment of modified Scots pine wood with straight chain alkyl anhydrides demonstrates an improvement of wood to biological attack (Suttie *et al.*, 1997).

A research by Okino *et al.* (1998) showed acetylated pine and eucalyptus flakeboards are in a high resistance class against white rot and brown rot fungi.

Another research by Ohkoshi *et al.* (1999) indicated that the decay by brown rot fungus became inhibited by acetylation at a WPG of more than 10% and mass loss due to decay became zero at a WPG of

about 20%. The weight loss due to white rot fungus decreased slowly with the increase in WPG, reaching zero at a WPG of about 12%.

Ibach *et al.* (2000) tested bioresistance of different acetylated wood against termites, brown rot and white rot fungi. Results showed acetylation was effective against brown rot fungus (*Tyromyces palustris*) and the white rot fungus (*Coriolus versicolor*).

b.3. Termites

Acetylation improved resistance to decay at WPG 6-20% and subterranean termites at WPG 13-18% (Kumar, 1994).

An increased resistance to termites for acetylated wood as compared with unmodified wood was reported by Hadi *et al.* (1995).

A research by Ibach *et al.* (2000) showed resistance of different acetylated wood against subterranean termites (*Coptotermes gestroi*) and dry wood termites (*Cryptotermes cynocephalus*) in laboratory testing.

Different researches on wood composites showed the positive effect of acetylation on their bioresistance. For example, finished and unfinished acetylated fiberboards (WPG 15%) were tested and results showed less mildew growth after outdoor weathering when compared to untreated boards (Feist *et al.*, 1991).

Rowell *et al.* (1997) tested acetylated composites in-ground stakes world-widely and reported that after three years of testing, acetylation of wood provides excellent protection against fungal attack and minimizes swelling.

Westin (1998) tested resistance of acetylated wood fiber composites against biological decay in a worldwide field test where 30cm long fiberboard stakes were half buried in soil. Results indicated that after three years of testing, most of the control stakes had failed due to heavy decay, while most of the acetylated composite stakes were perfectly sound and shown no sign of decay.

Westin (1998) also investigated high performance composites from modified wood fibers, which were prepared with two methods, acetylation and Kraft lignin, and compared them for their resistance to wood decaying microorganisms (fungi, bacteria and insects). Results showed a very high biological decay resistance against them.

c. Weathering & UV resistance

Wood exposed to accelerated weathering will be rapidly changed in its color, and surface becomes rough and checks and cracks appear. Water in combination with UV light has a great impact on the weathering and surface degradation of wood. The UV radiation causes photochemical degradation mainly in lignin polymers in cell walls. As lignin is degraded by UV radiation, water washes away degradation products and subsequently losses surface cellulose fibers, which cause wood to be deteriorated (Feist *et al.*, 1991).

Plackett *et al.* (1992) reported an improvement in color stability and weather resistance in acetylated Radiate Pine veneers when exposed to accelerated weathering for 3000 hours. Also acetylated Radiate Pine wood veneers showed only a slightly lighter color as compared with unmodified controls when exposed to natural weathering for 28 weeks (Dunningham *et al*., 1992).

Acetylated wood color was assessed by exposing to UV light during 56 day of exposure. Change in color and reduction of reflectance signaled an interaction of acetylated wood with electromagnetic energy. Acetylated wood exhibited a color stabilization effect better than non-acetylated wood after the initial 28 days of irradiation. Its stabilization effect steadily diminished and discoloration started (Hon, 1995).

Evans *et al.* (2000) studied the effect of natural weathering on acetylated Scots Pine veneers (WPGs 5, 10, 15 and 20%). Veneers were acetylated to low WPGs (5 and 10%) showed a greater loss in mass and tensile strength due to increased delignification and depolymerisation of cellulose than similarly exposed untreated controls. But acetylation to WPG 20% restricted the loss of veneer mass and holocellulose during exposure.

Sander and Koch (2001) investigated UV-absorption of acetylated and hydrothermally modified Norway spruce wood and observed a less UV-absorption in acetylated wood due to superficial leaching of lignin during acetylation.

Weathering and UV resistance are also experienced with acetylated wood composites. Finished and unfinished fiberboards were prepared from untreated and acetylated (WPG 15%) aspen fibers and exposed to accelerated outdoor weathering and was found in both acetylated fiberboards, less mildew growth after outdoor weathering compared with untreated boards (Feist *et al.*, 1991).

Researches also showed that acetylated fiberboards were smoother than untreated ones. Also a 50% reduction in erosion for acetylated aspen fiberboards compared with unmodified boards was reported after 700 hours of accelerated weathering. It is believed that the reduction in weathering effects in acetylated aspen may be a result of modification in both lignin and hemicelluloses (Plackett *et al.*, 1996).

d. Mechanical properties

Dreher *et al.* (1964) found in acetylated Ponderosa pine, Red oak and Sugar maple that dimensional stability, specific gravity, compression strength perpendicular to the grain, ball hardness, and fiber stress at proportional limit and work to proportional limit are higher than the same parameters in non-acetylated woods. But MOE, MOR and shear strength parallel to grain are less than those in non-acetylated woods.

Dhamodaran (1995) acetylated Rubberwood to WPGs 18-22% and studied the mechanical properties, dimensional stability and biological resistance. He found an increase about 85-88% antishrink efficiency. But modulus of rupture (MOR) decreases slightly as the WPG increases. MOR of treated rubber wood is slightly less than the untreated wood, but the difference is not large enough to affect the utilization value. However, maximum compressive strength (MCS) is not at all affected by acetylation.

Ramsden *et al.* (1997) acetylated Scots pine wood blocks for different periods of time by acetic anhydride in xylene and tested tensile modulus, hydrophobicity and dimensional stability. Observation showed that the acetylation process significantly reduces tensile modulus of wood compared to its untreated state.

Larsson (1998) studied influence of acetylation on selected wood properties in Scandinavian pine and Spruce wood to predict the behavior of acetylated wood when it is used in different applications. Results showed only minor differences in both bending strength and modulus of elasticity for acetylated wood when compared with unmodified wood. Briell's hardness also was found to increase and tendency of wood to deform under a cycle relative humidity condition was greatly reduced due to acetylation. Acetylated wood also showed strong adhesive bonds with conventional wood adhesives. When laminated wood was tested under wet conditions, the bond strength was higher in acetylated laminated wood than unmodified.

The effect of acetylation on wood composites was also studied and reported by different authors. For example, Rowell *et al.* (1989) prepared particle boards from Sraya acetylated wood particles (WPGs 15-17%) and compared their dimensional stability and mechanical properties (moduli of elasticity and rupture and screw holding). Results showed a slight reduced modulus of elasticity and rupture in acetylated boards. Internal bond strength was reduced by about 30% in acetylated boards. But screw-holding capacity was the same with non-acetylated particleboards.

Rowell *et al.* (1995) made low-density fiberboards from acetylated aspen fibers and observed a low modulus value in lower densities compared with untreated ones. Also Rowell *et al.* (1991) reported that fiberboards made from acetylated bagasse fibers (WPG 17%) had EMCs $^{1}/_{3}$ of controls at all relative humidities and also internal bond strength was higher in acetylated fiberboards, while their moduli of rupture and elasticity were slightly lower than non-acetylated.

The effect of acetylation on mechanical and physical properties of dry-process hardboards made from aspen and Southern pine was investigated by Chow *et al.* (1996). Test results indicated that MOR and MOE of acetylated hardboards were decreased due to acetylation. Also tensile stress parallel to face and internal bond was lower than untreated boards.

Westin (1998) investigated high performance composites from modified wood fibers. He used two modification methods, acetylation and Kraft lignin, and reported maintaining of mechanical properties

(internal bonding strength, IBS) during and after cyclic climate aging (85%). Also he reported that composites based on acetylated wood fibers are truly high performance.

It is reported that there are reductions in the MOE and MOR values of acetylated flakeboards compared with untreated boards produced from *Gmelina arborea* and *Picea sitchensis*. They are maybe due to the acetylated flakes being rendered more brittle (Fuwape & Oyagade, 2000).

e. Acoustic properties

One of the crucial characteristics of wood in making musical instruments is its acoustic property. Main factors affecting the acoustic properties of wood are grain angle, temperature, moisture content, density and extractives. As wood is a hygroscopic material, the dimensions of wood products change when the ambient humidity changes. This affects the tone quality of wooden musical instruments. Because, when moisture content increases, the acoustic properties of wood such as specific dynamic Young's modulus (E'/g) and internal friction (Q^{-1}), are reduced or dulled. The absorption of water molecules between the wood cell wall polymers acts as a plasticizer to loosen the cell wall microstructure. The decrease in cohesive forces in the cell wall also enhances the deformation of wooden parts under stress. Stress possibly occurs due to forces in the strings of guitars and violins and the pin block in a piano. So stabilizing the dimensional and acoustic properties of wooden musical instrument by delaying the absorption of moisture in wood is of great importance (Yano *et al*., 1993; Chang *et al.*, 2000).

Yano *et al.* (1993) studied the acoustic properties of acetylated Sitka spruce wood and reported that acetylation reduces slightly sound velocity (about 5%) and sound absorption in wood. But it increases acoustic and dimensional stability of wood by reducing moisture absorption in cell walls.

1.4. Other types of wood modification

There other types of wood modification that have not established based on chemical methods. They are categorized based on the methods, which are used. The most important types of wood modification are thermal wood modification and enzymatic wood modification,

1.4.1. Thermal wood modification

Wood is placed in a kiln and gradually heated up to between 180°C to 280°C, irreversibly altering its molecular structure and mineralizing the cellulose, to produce a kind of instant "*fossilized wood*" which still remain workable. Polysaccharides inside the wood break up to a form, which rot fungi can not use. Heat treatment lasts 24-72 hours with cooling and final stabilizing. The temperature of a batch of wood is raised gradually in a drying kiln and then lowered slowly during the cooling phase, to keep the wood from splitting. Industrially heat-treated wood is called "fossilized wood", "baked wood", "thermo wood", "retified wood" and "cooked wood". Different processes in thermal wood modification have been introduced by industries by now, which are as follow:

b. PLATO-Process (PLATO BV, The Netherlands)

The PLATO-process patented by Ruyter (1989). Recently Tjeerdsma *et al.* (1998 a&b; 2000) and Boonstra *et al.* (1998) have used this process to treat wood thermally. This thermal treatment (which is called "PLATO- process" in SHR, The Netherlands) is the use of different steps of treatment and combining successively a hydrothermolysis step with a dry curing[2] step. The impact of hydrothermolysis in the PLATO-treatment results in the occurrence of many chemical transformations. The presence of abundant moisture in the woody cell wall during the hydrothermolysis provokes increased reactivity of cell wall components under comparable low temperature. In order to reach a selective degree of depolymerisation of the hemicelluloses during the hydrothermolysis, relative mild conditions can be applied and unwanted side reactions, which can influence the mechanical properties negatively, can be minimized. The PLATO-process consists of two stages with an intermediate drying operation. In the first step (hydrothermolysis) of the process, green or air dried wood, is treated at temperatures typically between

[2] ***a)*** Process of heating or otherwise treating a rubber or plastic compound to convert it from a thermoplastic or fluid material into the solid, relatively heat-sensitive state desired in the commercial product. When heating is employed, the process is called *vulcanization*. ***b)*** The final drying stage where the paint reaches maximum strength (refer to: Babylon online dictionary; http://www.babylon.com/). In thermal wood treatment, it refers to the stage where wood has lost its moisture content and is going to reach its highest strength and stability.

160°C-190°C under increased pressure. A conventional wood drying process is used to dry the treated wood to low moisture content (ca. 10%). In the second step (curing), the dry intermediate product is heated to temperature typically between 170°C-190°C (Militz & Tjeerdsma, 2002).

c. Retification process (NOW New Option Wood, France)

The "retification" process of wood was developed by "Ecole des Mines" of Sain Etienne within last ten years. It consists of a thermal treatment of dry wood (MC about 12%) between 200°C and 260°C with an atmosphere poor in oxygen (less than 2% oxygen) in presence of nitrogen. The treatment process starts under temperature between 200 and 260°C for short period of time with lack of air. The duration of treatment depends on the size of wood material; for example for a 1cm thickness, the treatment duration lasts about 5 hours; 30min at 280°C, 1h at 250°C and 2h at 230°C and approximately 9h for a material with 27mm thickness (Militz, 2002; Yvan, 1982; Dirol & Guyonnet, 1993; Troya & Navarrete, 1994; Bourgois *et al.*, 1989; Bourgois *et al.*, 1990; Vernois, 2002).

d. Le Bois perdure (BCI-MBS, France)

Instead starting from dry wood in retification, the process starts from fresh wood. The first step is rapid drying process and heating up to 230°C under steam atomosphere (Militz, 2002; Vernois, 2002).

e. VTT process (Thermowood, Finland)

This process is licensed to Finnish Thermowood Association. The Thermowood is based on heating wood at high temperature (180-250°C) by using a water vapor as shielding gas. While heating wood at temperatures over 200°C, wood undergoes a large number of chemical changes, like degradation of wood hemicelluloses. VTT method differs from other methods that use nitrogen as shielding gas and some processes are done under pressure. This process has been divided into three main phases:

Phase 1- Temperature increase and high temperature kilning- Kiln temperature is raised at a rapid speed using heat and steam to level of around 100°C. Thereafter the temperature is increased steadily to 130°C during which the high temperature drying takes place and the moisture content in the wood reduces to nearly zero.

Phase 2- Intensive heat treatment- On the high temperature kiln drying has taken place the temperature inside the kiln is increased to level between 185-230°C, once the target level has been reached the temperature remains constant for 2-3 hours depending on the end use application.

Phase 3- Cooling and moisture conditioning- The final stage is to lower the temperature down using water spray system then once the temperature has reached 80-90°C remoisturising and conditioning takes place to bring the wood moisture content to a useable level over 4%. When raising or decreasing the temperature a special adjustment system is used in order to prevent surface and inside cracking. The wood's internal temperature regulates the temperature rise in the kiln. The difference between kiln and wood temperature is depending on the dimensions of the wood specimens (Mayes & Oksanen, 2002).

If the process starts from green wood, the wood can be dried in a very rapid steam drying process. Rapid drying is possible because any care for the color changes is not necessary and also resins will anyway flow from the wood in heat treatment process (Militz, 2002; Jämsä &Viitaniemi, 2000; Syrjänen & Oy, 2000).

f. OHT-process (oil-heat treatment, Menz Holz, Germany)

Heat treatment usually takes place in an inert gas atmosphere at temperatures between 180°C and 260°C. The boiling points of many natural oils and resins are higher than the temperature required for the heat treatment of wood. This opens up the opinion of the thermal treatment of wood in hot bath. Improvements in various wood characteristics can be expected from the application of oil-heat treatment as compared with heat treatment in a gaseous atmosphere, due to the behavior of oils in conjunction with effect of heat.

Principally, this process is performed in a closed vessel. After loading the process vessel with wood, hot oil is pumped from the stock vessel into the process vessel where the hot oil is kept at high temperatures circulating around the wood. Before unloading the process vessel the hot oil is pumped back into the stock vessel.

For different degrees of upgrading, different temperatures are used. To obtain maximum durability and minimum oil consumption the process is operated at 220°C. To obtain maximum durability and maximum strength temperatures between 180°C and 200°C are used plus a controlled oil uptake.

It proved to be necessary to keep the desired process temperature (for example 220°C) for 2-4 hours in the middle of the wooden pieces to be treated. Additional time for heating up and cooling down is necessary, depending on the dimension of the wood. Typical process duration for a whole treatment cycle (including heating up and cooling down) for logs with cross sections of 100mm×100mm and 4m length is 18 hours.

The heating medium is crude vegetable oil. For example rapeseed (canola), linseed oil, sunflower oil, soybean oil, tall oil or even its derivatives. The serves for fast and equal transfer of heat to the wood, providing the same heat conditions all over the whole vessel and also a perfect separation of oxygen from wood. Natural oils lend themselves to the oil-heat improvement of wood from an environment point of view and because of their physical point of view and chemical properties. As renewable raw materials, they are CO_2 neutral. Between other types of oils that were used, linseed oil proved to be unproblematic though the smell that develops during the heat treatment may be a drawback. The smoke point and the tendency to polymerization are also important for the drying of the oil in the wood and for the stability of the respective oil batch. The ability of the oil in the wood to withstand heating to a minimum temperature of 230 °C is a prerequisite. The consistency and color of the oil change during heat treatment. The oil becomes thicker because volatile components evaporate, the products arising from decomposition of the wood accumulates in the oil and its composition. This obviously leads to improve setting of the oils (Rap & Sailer, 2000; Thévenon, 2002).

Thermal wood modification has different effects on wood properties; such as increase in its dimensional stability (Yildiz, 2002; Bengtsson *et al.*, 2002; Vernois, 2000; Sailer *et al.*, 2000; Rap & Sailer, 2000; Mayes & Oksanen, 2002; Boonstra *et al.*, 1998; Militz & Tjeerdsma, 2000) considerable increase in bioresistance against fungi (Welzbacher & Rapp, 2002; Matsuka *et al.*, 2002; Viitanen *et al.*, 1994; Kamdem *et al.*, 1999; Tjeerdsma *et al.*, 1998; Tjeerdsma *et al.*, 2000; Mayes & Oksanen, 2002; Rap & Sailer, 2000; Sailer *et al.*, 2000; Militz & Tjeerdsma, 2000; Boonstra *et al.*, 1998; Viitanen *et al.*, 1994; Dirol & Guyonnet, 1993) and some times slight reduction in mechanical properties (Bengtsson *et al.*, 2002; Sailer *et al.*, 2000; Kamdem *et al.*, 1999 & 2002; Militz, 2002; Jämsä &Viitaniemi, 2000; Syrjänen & Oy, 2000; Militz & Tjeerdsma, 2000).

1.4.2. Enzymatic wood modification

Because of the growing utilization of renewable raw materials, the technical use of lignocellulosic fibers from wood and other annual plant materials is becoming increasingly important. For example as a technical problem, the synthetic adhesives used in manufacturing fiberboards cause both emission and waste problems. Alternatively, enzyme systems naturally responsible for biosynthesis and coupling of wood cells can be used.

Wood composites, especially fiberboards, are used to produce both furniture and indoor installations. Fiberboard is made by triturating wood into single fibers and then adding synthetic adhesive and pressing the fibers together at approximately 200°C. The synthetic adhesives used in this production are typically based on the substances formaldehyde, urea, phenol and melamine. As the use of formaldehyde is particularly problematic, only a very low amount of this substance may be released subsequent to production. The use of synthetic adhesives also means that possible fiberboard waste may not be burnt, but most be disposed of as special waste.

Consequently, new methods are needed for coupling and bonding wood fibers. For a number of years, experiments have been conducted with new adhesives based on waste material from the paper industry, etc. But developments in biotechnology have given the industries the possibility of using the same enzyme system as nature uses to create and couple cells and cell walls in wood. This is an interested way to produce a product that is more environment-friendly than fiberboards made with synthetic adhesives. At least, the first advantage of using enzymes rather than synthetic adhesives to bond the boards is that they pose no emission or waste problem, etc.

Wood consists of three main components, cellulose, hemicelluloses and lignin. Much of lignin content is found in the outer layer of the cell walls, where it couples the individual cells and stiffens the cell wall. In

this purpose, use of enzyme those catalyses the coupling of lignin between cell walls to couple single fibers to a board. This enzyme is called *laccase*. Laccase acts to aerate or oxidize lignin. Aerating removes electrons, creating single electrons called radicals. Since electrons preferably occur in pairs, the radicals will try to pair up, thus creating chemical bonds (gluing) between the individual fibers. Using laccase for oxidation causes stable radicals to form in the lignin contained in the fibers cell walls (Felby *et al.*, 1997; Unbehaun *et al.*, 2000).

Laccase is recently known to use for oxidation of phenolic compounds in wood. During the reaction, phenoxy radicals are formed in lignin components while oxygen is reduced to water. The reaction mechanism can be described by a parallel mechanism of direct oxidation of lignin on the fiber surface and a phenol/phenoxy cyclic mediator process of dissolved phenolic compounds in the suspension liquid.

1.5. Aims of this thesis

Concerning the above review on literatures, there are still unknowns about the acetylation. This research has been conducted to answer some questions about the biological degradation of the acetylated wood.

- Could acetylated wood be influenced by soil microorganisms over a long period efficacy?

Acetylated wood has not been tested over longer period of time in real field and soil beds to find out the changes of mass and dynamic modulus of elasticity (MOE_{dyn}). According to a time course study, any changes in mass and MOE_{dyn} in acetylated wood were studied during a standard soil bed test and the results are discussed in chapter 2.

- Does acetylation protect wood from chemical alterations due to biological attack?

Any biological attack in wood is followed by chemical alterations. No investigation was carried out so far about the chemical alteration in the acetylated wood. Based on a time course study, chemical analyses were carried out on the soil bed tested acetylated wood and the results are presented and discussed in chapter 2.

- How do microorganisms attack acetylated wood? And where are their attacking points in cell walls? Is there any difference between microbial attacks in acetylated wood with non-acetylated?

Microorganisms, fungi and bacteria belong to important biological attacking factors of wood. Monitoring of microbial attack in acetylated wood was one of the interests to help gathering more knowledge about their activities in acetylated wood. Different microscopical techniques, light and scanning electron microscopy, were used to evaluate different types of biological attack in soil exposed acetylated wood and fungal trials. Chapters 2, 3 and 4 discuss the microbial attack in soil exposed acetylated wood, real field and fungal trials respectively with different acetylated woods.

- Could using biological indicators demonstrate the microbial metabolic activities in acetylated wood?

Biological activities occur with some metabolic sequences. Concerning this fact that wood is attacked by parasite microorganisms and is used as a carbon source material, heat is released due to their metabolisms. Heat production and released energy were measured by microcalorimetry to evaluate soil microorganisms and fungal activities in acetylated wood to find out any differences in their metabolic activities. The obtained results about their bioactivities are discussed in chapters 2 and 5.

- What is the difference between fungal colonization and biomass in acetylated and non-acetylated wood?

There are different techniques to measure fungal biomass in wood. For this purpose a fungal bio-indicator, ergosterol and fluorescein diacetate, have been used to measure fungal biomass in the acetylated wood. Ergosterol was used for soil fungi and white rot fungus *Trametes versicolor*. The results are explained in chapters 2 and 5 respectively. Fluorescein diacetate was used just for white rot fungus and the obtained results are presented in chapter 5.

- Could enzymatic activities relate to microbial attack in acetylated wood?

In chapter 5 enzymatic activities are investigated by measuring cellulases, endoglucanases, xylanase, laccase and manganese peroxidase.

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Chapter 2

Soil bed trials with acetylated wood

Material and methods

2.1. Sample preparation

Small mini-stakes in sizes 5×5×110mm were prepared from Beech (*Fagus sylvatica*) and Scots pine (*Pinus sylvestris*) sapwoods. They were initially dried for 24 hours at 60°C in an oven to reach equal moisture contents before acetylation. Uniform acetylation obtains at lower wood moisture content (Beckers & Militz, 1994a). About 10 mini-stakes of each batch were dried at 103±2°C and their moisture contents were determined to calculate the dry weight of all mini-stakes before the acetylation. Different batches of mini-stakes were prepared separately for soil bed, white and brown rot trials before the acetylation and weights were measured for all mini-stakes prior to the acetylation.

2.2. Acetylation

The prepared mini-stakes were treated in a reactor (fig. 2-1) with acetic anhydride under different process conditions (table 2-1) to achieve different weight gain percentages (table 2-2). The acetylation was carried out under vacuum condition to reach a uniform penetration of acetic anhydride. The weight of wood samples gains after the acetylation and it is usually indicated by weight percent gain (WPG).

After acetylation, the mini-stakes were soaked in de-mineralized water to convert acetic anhydride to acetic acid within few days. Afterwards, they were dried at 120°C to remove the remained acetic acid. Dried weight of all mini-stakes was also determined to calculate the WPGs (equation 2-1). The average of WPGs was calculated for each batch of experimental samples.

Fig. 2-1. The acetylation reactor (by courtesy of SHR, The Netherlands).

Table 2-1. Operated conditions for acetylation of Beech and Scots Pine mini-stakes

Acetylation Process	Prevacuum		Reaction solution		Reaction condition		Postvacuum	
	Period	Pressure	Acetic anhydride	Acetic acid	Temperature	Time	Period	Pressure
	(min)	(barg)			(°C)	(min)	(min)	(barg)
A	-	-	-	-	-	-	-	-
B	30	-0.97	25%	75%	80	180	90	-0.97
C	30	-0.97	75%	25%	80	180	90	-0.97
D	30	-0.97	50%	50%	120	180	90	-0.97
E	30	-0.99	100%	0%	120	180	60	-0.99
Post treatment:								
All mini-stakes were soaked in de-mineralized water to convert the acetic anhydride to acetic acid.								
Afterwards the mini-stakes were dried at 120 °C to remove the remained acetic acid.								

Eq. 2-1. $$WPG\ (\%) = (W_{act} - W_{od}) / W_{od}.100$$

Where:
WPG= Weight percent gain (%)
$\mathbf{W_{act}}$= Oven dried weight after acetylation (g)
$\mathbf{W_{od}}$= Oven dried weight before acetylation (g)

2.3. Soil bed trials

2.3.1. Sample preparation

Acetylated mini-stakes (14 samples) were coded according to sampling time intervals and average of WPGs for batches was determined (table 2-2).

Table 2-2. Average of WPGs in Beech and Scots pine wood

Acetylation process	Weight percent gain	
	Beech	Scots pine
	%	%
A	0	0
B	1.84	2.43
C	6.72	9.13
D	8.33	10.22
E	17.43	19.46

The initial dry weights and dynamic moduli of elasticity (MOE_{dyn}) were determined for all mini-stakes by a Grindo*Sonic* MK5 before experiment.

2.3.2. Determination of dynamic Modulus of Elasticity (MOE_{dyn})

Different test methods exist for the determination of modulus of elasticity (MOE). These methods can be divided into two groups. The first one comprising static methods, is the most conventionally used and described in most standards for MOE determination. The second groups are dynamic methods, which are based on resonant vibration excitation or ultrasonic pulse excitation. Dynamic methods provide some advantages compared to static methods. The tests are non-destructive in nature, feasible to perform on-site measurements and no laboratory stand equipment is needed. In addition, the use of vibration techniques enables considerable reduction in testing time and labor costs (Machek *et al.*, 1998a&b)

Fig. 2-2. Grindo*Sonic* MK5 'Industrial' (J.W. Lemmens, N.V. Leuven; Belgium);
Ham: Hammer, Sup: Support, Mic: Microphone, Mon: Monitor

Grindo*Sonic* MK5 Industrial (fig. 2-2) was used for MOE_{dyn} determination (Machek *et al.*, 1997; Machek *et al.*, 1998a&b; Machek *et al.*, 2001; Machek *et al.*, unpublished). It operates based on vibration

energy (fig. 2-3). Each specimen is supported by two triangle polystyrene supports (Sup) horizontally and placed on its nodal points of the fundamental resonance frequency. The points are located at a same distance from both ends of specimen. It is equal to 0.224 × L of each end (L is the length of specimen). The vibration initiates into the specimen through a hammer (Ham) impact on the middle length on top surface (radial direction). A very sensitive sensor (Mic) transfers the signals into the device and it shows them as frequency in Hz on its monitor (Mon) (fig. 2-2 & 3).

The MOE_{dyn} of mini-stakes was calculated by equation 2-2.

Eq. 2-2 $$MOE_{dyn} = \{(4.\pi^2.L^4.f^2.\rho.A)/(m_1^4.I)\}.\{1 + I/(L^2.A).K_1\}$$

$$I = b.h^3/12$$

Where:

MOE_{dyn} = Dynamic modulus of elasticity (N/mm^2)

I= Moment of Inertia (mm^4)

A= Area of cross section (mm^2)

f= Frequency (KHz)

ρ= Density (gr/mm^3)

L= Length (mm)

K_1= 49.48

m_1= 4.72

I= Moment of inertia (mm^4)

b= Width (mm)

h= Height (mm)

Fig. 2-3 shows distribution of initiated impact energy on impact point (ip) through the specimen.

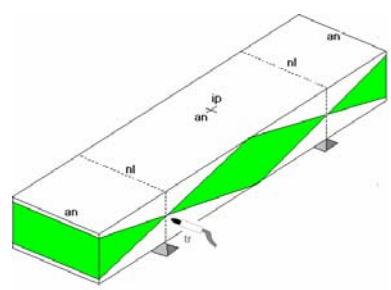


Fig. 2-3. Schematic representation of the vibration test. an: antinode; nl: nodal line; ip: impact point; tr: transducer

2.3.3. Soil bed preparation

According to ENV 807 (1993) soil beds were prepared in plastic containers (43 × 32 × 21 cm). The containers were supplied with different soil layers: from bottom to top; 20mm of gravel, 20mm of river sand, 150mm of loam-based horticultural soil (John Innes II). Water holding capacity (WHC) was determined and soil moisture was adjusted to 95% of its WHC. Afterwards for every sampling term, 14 mini-stakes were planted randomly into the soils vertically with 20mm of their length protruding above surface of soil and with a minimum 20mm between adjacent specimens and from sides of container (fig. 2-4). The prepared soil beds were kept at a controlled condition with temperature 26±1°C and relative humidity 65±5%.

Sampling interval was considered for 2 month except for first sampling term that was just for one month. Any losses in mass and dynamic modulus of elasticity (MOE_{dyn}) were measured as explained above.

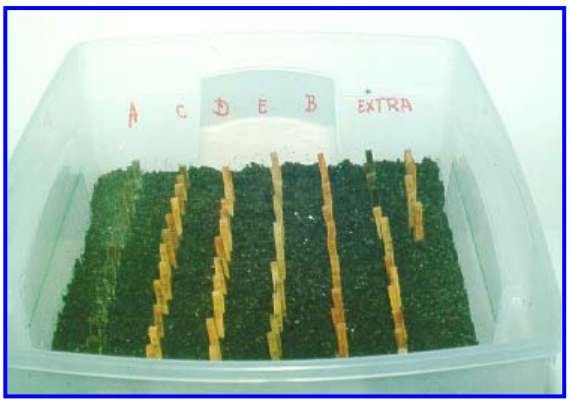


Fig 2-4. A soil bed with planted mini-stakes in randomized WPG (A-E).

2.3.4. Chemical analysis

Microorganisms change the chemical composition of wood during decay (Zabel & Morrell, 1992). This chemical alteration is unknown in acetylated wood. It is not known if the cell wall polymers of acetylated wood are degraded by fungi in the same manner as untreated wood. For this purpose, lignin content, holocellulose and α-cellulose were determined before and during attack period.

2.3.4.1. Extractives free wood preparation

The extractives are group of cell wall chemicals mainly consisting of fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, rosin, waxes, etc. These chemicals exist as monomers, dimers, and polymers. They derive their name as chemicals that are removed by one of several extraction procedures. The presence of extractives is erroneous and it should be removed from wood to avoid any calculation errors.

The mini-stakes of each sampling term were pooled and ground with a mill Retsch ZM100 and sieved with a screen to pass a mesh-40 (0.4mm) to obtain same sizes of particles and remove fine particles, according to TAPPI T264 (TAPPI 1997). Milled woods samples were divided into two portions as replicates and used for chemical analysis.

Ethanol and an ethanol-cyclohexane mixture or acetone, remove the materials which are not part of the wood substance (extractives) or which may interfere with some analysis. Therefore, wood extractives were removed by a mixture of ethanol-cyclohexane (1:2) for 8 hours, ethanol 95% for 4 hours and hot water for 1 hour according to TAPPI T264 (TAPPI 1997) to prepare extractive free wood for other chemical determinations.

2.3.4.2. Klason lignin

Lignin and carbohydrates are the main components in the cell walls. Wood normally contains of 20-30% lignin. Lignin could be removed or modified by different types of fungi and bacteria. Changes in lignin percentage of acetylated wood by microorganisms are still unknown during the wood degradation.

According to TAPPI T222, Klason lignin was determined in extractive free wood. A portion of milled wood samples (1 g) were weighed for each WPG and sulfuric acid (15ml) was added gently during 15min in three steps (every 5min) and mixed carefully in an ice-water bath till color changed to dark black or brown. Samples were kept at room temperature for 2 hours. Afterwards, they were transferred to Erlenmeyers flasks containing diluted sulfuric acid (3%) and heated up at boiling temperature for 4 hours (TAPPI 1998). They were cooled down and permitted prepared lignin to precipitate at room temperature overnight. Supernatants were decanted and Klason lignin was filtered and dried at 105±3°C (O/N) and weighed. Lignin content was calculated based on oven dry weight. Parallel samples of milled wood were weighed and dried before lignin determination to calculate wood moisture content.

2.3.4.3. Holocellulose

The combination of cellulose and hemicelluloses are called “holocellulose” and usually accounts for 65–70% of the plant dry weight. These polymers are made up of simple sugars, mainly, D-glucose, D-

mannose, D-galactose, D-xylose, L-arabinose, D-glucuronic acid, and lesser amounts of other sugars such as L-rhamnose and D-fucose. These sugars are the first accessible nutrients for microorganisms. Any biological decay affects the amount of holocellulose in decayed wood. There is no report to indicate the variation of holocellulose in acetylated wood.

A part of extractive free wood was used to determine the holocellulose according to method of Wise *et al.* (1946). About 2 g extractive free wood was placed in 100ml Erlenmeyer and 80ml distilled water was added. Also 0.25ml acetic acid (100%) and 0.75g sodium chlorite ($NaClO_2$) were added into the Erlenmeyers and closed with a glass bubble. They were kept at 80°C for 1 hour in hot water bath. Again the same amount of acetic acid and sodium chlorite were added to the Erlenmeyers. The additions were repeated three times for beech and four times for Scots pine wood and were kept at 80°C. They were finally cooled in an ice-water bath, filtered with a glass filter and washed with 100ml distilled ice-water and 25ml acetone. The filtered samples were dried at 105±3°C (O/N) and weighed.

2.3.4.4. α-Cellulose

Cellulose is the main component of plant cell walls. This carbohydrate influences by fungi and bacteria during degradation. The alteration of cellulose is unknown in acetylated wood.

Oven dried samples, which were obtained from holocellulose determination, were used for α-cellulose determination according to TAPPI T203 (TAPPI 1988). They were weighed again and placed in 100ml Erlenmeyers. Sodium hydroxide (NaOH 17.5%) 75ml was added into the Erlenmeyers and kept at 20°C for 2 hours, shook every 15 minutes and then filtered by glass filter. Washing was carried out with 25ml sodium hydroxide (NaOH 17.5%), 150ml distilled water, 25ml acetic acid (10%) and 25ml acetone respectively. Finally, they were dried at 105±3°C (O/N) and weighed.

The measured amounts of chemical components were corrected based on following equation (Ohkoshi *et al.*, 1999). Because, the chemical contents were measured above refer to the quantity of the chemicals based on the weight of decayed wood. Therefore, to obtain the chemical contents in decayed wood based on the weight before decay, the mass losses due to decay has to be taken into account. Moreover, because the acetyl groups were probably removed by sulfuric acid used during the preparation of Klason lignin, the weight gains due to acetylation must be counted.

Eq. 2-3 ***Corrected amount = Measured amount × (1-mass loss/100) × (1- WPG/100)***

2.3.4.5. IR spectroscopy

Infrared spectroscopy is a very useful tool for obtaining rapid information about the structure of wood constituents and chemical changes taking place in wood due to various treatments or biological degradation. Fourier transform infrared (**FTIR**) spectroscopy has been used for wood surface characterization, estimation of lignin and carbohydrate contents in wood and lignocellulosics. Attenuated Total Reflection (**ATR**) Infrared Spectroscopy has an advantage over other methods. Because it is a quick, easier, and nondestructive method, and the structure of wood is maintained when spectra are measured directly from solid wood surfaces. The alteration in the acetylated wood and exposed wood to soil beds were analyzed by ATR method, due to its easiness and also quickness.

IR spectra of acetylated unexposed and soil-exposed wood and its major components, α-cellulose and Klason lignin, were studied by ATR. For this purpose, powder of acetylated milled wood and also its isolates, Klason lignin and α-cellulose, were used. IR spectra were collected directly from wood powder, lignin and α-cellulose isolates on detector prism (fig. 2-5). Spectra were recorded using a Bruker Vectra 22 FTIR Spectrometer equipped with a DuraSampl*IR* II™ detector. All spectra were taken at a spectral resolution of $4cm^{-1}$. Sample and background scans were measured with 30 scans. Background spectra were collected using an empty collector. A rubber band method was used for baseline correction. The band for CO_2 was removed to make a suitable baseline correction.

Fig. 2-5. Collecting IR spectra by FTIR spectrometer

2.3.5. Microscopy

Biological decay patterns of soil microorganisms in different WPGs were studied under microscopes. For this purpose, two specimens of each WPG in every sampling period were considered for microscopy. Therefore, two mini-stakes were not measured for determination of MOE_{dyn} and mass losses after exposing to soil microorganisms. After taking the samples from the soils, they were kept in a refrigerator under temperature 4°C to cease any fungal and bacterial activities in decayed wood before any microscopy.

2.3.5.1. Light microscopy

Mini-blocks in sizes about 5 × 5 × 10 mm were cut from the end of soil exposed side of each mini-stake after leaving one centimeter to the end. Then cross, tangential and radial sections (10μm) were prepared from fresh specimens and stained with a mixture of safranin and astra blue immediately for 10 minutes (Klassen *et al*, 2000; Srebotnik & Messner, 1994) and mounted with Kaiser's Glycerol Gelatin. Remained microtome trimmed and flattened mini-blocks were cut into small sizes and used for electron microscopy.

2.3.5.2. Scanning Electron Microscopy

The specimens were fixed with 3% glutaraldehyde in 0.1M phosphate cacodylate buffer overnight. Then they were washed with the same buffer three times for 30 minutes. Post-fixation was carried out with 1% osmium tetra-oxide in 0.1M phosphate cacodylate buffer for 4 hours. Fixed specimens with osmium tetraoxide were rinsed by distilled water three times for 30 minutes. Dehydration with ethanol series (10, 30, 50, 70, 80, 90 and three times 100%) was carried out for 30 minutes (each step). Critical point drying was applied with CO_2 at 42°C. The specimens were coated with Au/Pd at 0.4 torr pressure and 20mA for 3min. And finally they were examined under a Jeol JSM-5200 SEM at 15-20 kV.

2.3.6. Microcalorimetry

Fungal bioactivity may be assessed by various methods: mass loss of substrate, increased mass of fungus, linear hyphal growth, ergosterol content, chitin content, CO_2 production, heat production, O_2 consumption, or ATP concentration. As heat is released in all metabolic processes in plants, animals and microorganisms, isothermal calorimetry is a powerful method to study these processes. The released heat is proportional to the consumption of nutrients as long as the metabolic processes are constant. Isothermal calorimetry is the measurement of heat and heat production rates under essentially isothermal conditions (Bjurman & Wadsö, 2000; Xie *et al.*, 1997) and is a rapid method to determine bioactivity of microorganisms in decaying wood.

Heat production was monitored in a microcalorimeter of the heat conduction type with twin measuring background correction (2277 Thermal Activity Monitor, TAM: ThermoMetric, Jarvalla, Sweden), which is shown in fig. 2-6. Glass ampoules (2.5ml) with Teflon seals and stainless steel lids used as sample vessels (fig. 2-7). Wood specimens with sizes about 5 × 5 × 20 mm were cut from eroded end of soil exposed mini-stakes and were kept at room temperature (25°C) for 1 h to reach the room temperature and reactivate microorganisms. After conditioning, they were placed in the glass ampoules and held for few minutes at the equilibrium position and then lowered down into the channel gently. Internal calibration was carried out at 300μW in a static mode. The detection limit was ±1 μW and the background was an empty vessel. Average

heat production and produced energy rates were recorded at intervals of 10 seconds for 48 hours. The program DIGITAM 2.0 (SciTech Software, ThermoMetric, Jarvalla, Sweden) was used to record the heat production. Correction was carried out based on dry weight of the specimens (Bjurman & Wadsö, 2000; Xie *et al.*, 1997).

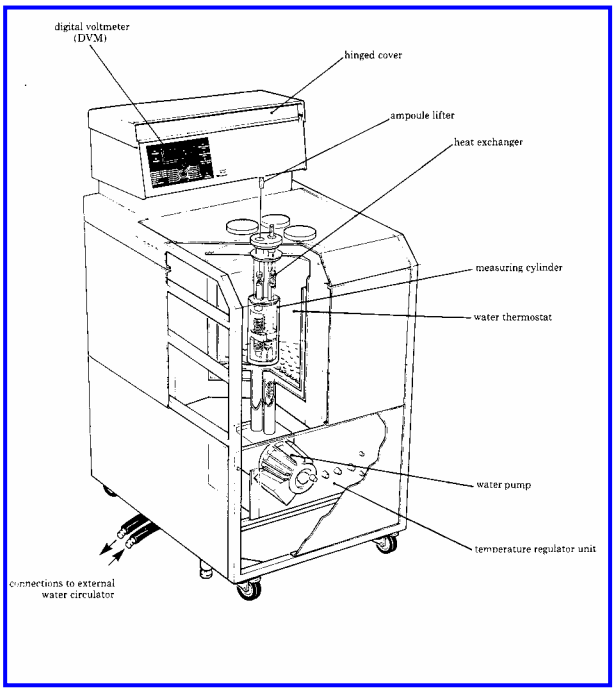


Fig. 2-6. Thermal Activity Monitor (TAM)

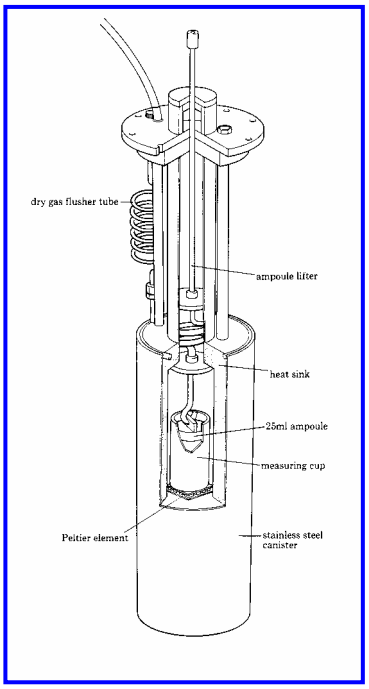


Fig. 2-7. Single measuring cylinder

2.3.7. Ergosterol assay in decayed wood

Ergosterol, 24,*β*-methylchlesta-5,7,trans-22-trien-3,*β*-ol, is a sterol as a prominent membrane component of most fungi, which has frequently been used as *fungal-index molecule* in natural substrates and it is not found in native wood (Encinas & Daniel, 1999; Weete, 1980; Karen, 1997). To estimate the fungal biomass in wood the ergosterol assay was used. Ergosterol analysis can also be used for detecting and quantifying fungal biomass of various biological materials; such as soils (West *et al.*, 1987), seeds (Seitz *et al.*, 1977) and mycorrhizas (Salmanovicz & Nylund, 1988). A chemical structure of ergosterol is shown below (fig. 2-8).

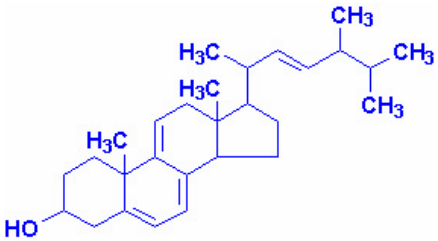


Fig. 2-8. Ergosterol (24,*β*-methylchlesta-5,7,trans-22-trien-3,*β*-ol)

Wood samples were pooled and crushed with a knife and transferred into homogenization vessels, and then homogenized in liquid nitrogen by Ultra-Thurrax. Homogenized wood was conditioned at room temperature to reach equal moisture content for about 24 hours. Their moisture contents were determined in an oven at 103±3°C to measure dry weights of samples. About 1.5g of samples were added into bottles containing below solution:

- KOH 10% in methanol 25ml
- 2,6,Di-tert-butyl-4-*P*-cresol (BHT)

= 2,6,Di-tert-butyl-4-methylphenol= ($C_6H_{24}O$) 200 PPM

They were heated at 60°C for 30 minutes in a water bath and then cooled down. About 4ml of extracted samples were transferred into Pyrex tubes (10ml) and 2ml n-Hexane was added to the tubes. They were mixed very well. Double distilled (4ml) water was added into the tubes and shaken for 10 minuets. Samples were centrifuged at 2000 rpm for 5 minuets. n-Hexane phase (1ml) in the tubes was pipetted into HPLC vials and dried in a vacuum chamber at 50°C. The congealed ergosterol in vials was dissolved in 300µl methanol (Braun-Lüllemann, 1995; Hendel & Marxsen, 2000) and run through the HPLC Hewlett-Packard Series 1100. Mobile phase was methanol 93% that was run with 1.5 ml/min automatically and 20 µl of sample was injected for each measurement with three replicates. Standard ergosterol (10, 50, 100 and 200 µl) was used to calibrate the HPLC after every 30 samples measuring. Retention time for ergosterol was between 10-11 minutes.

2.4. Results and discussion

2.4.1. Mass and MOE_{dyn} losses

A time course study during 300 days of soil bed tests in acetylated beech and Scots pine wood indicated decrease in weight loss at increased WPGs (fig. 2-9). Major mass losses were measured in non-acetylated wood which was 56.44% in beech and 27.6% in Scots pine wood after 300 days of exposure to soil beds; whereas no significant weight losses were determined in acetylated beech and pine wood at the highest weight percent gains. Results revealed that mass losses in both species decreased when their weight gains increased (fig 2-9A&B). Slight raise in mass was determined in highly acetylated beech and pine.

Fig. 2-10 represents variation of MOE_{dyn} in non-acetylated and acetylated beech and pine wood during 300 days of exposure to soil beds. Measurements showed that loss of MOE_{dyn} decreased when weight percent gains increased in both species. It was measured that non-acetylated wood lost its MOE_{dyn} rapidly. It was determined that beech and pine lost 84.14% and 71.92% of their MOE_{dyn} respectively after 300 days of exposure to soil beds; while no loss was measured in highly acetylated wood. There was only slight raise in MOE_{dyn} in both species instead.

Our measurements also reveled that MOE_{dyn} is a very good method to determine any attack in wood rapidly during early stages of attack. Corning the measurements, it was determined that beech and pine wood lost 7.43% and 3.43% of their weight and pine; while it was for MOE_{dyn} 30.49% and 11.53% in beech and pine wood respectively after 30 days of exposure to soil beds (figs 2-9 & 2-10).

Fig 2-11 represents losses of weigh and MOEdyn versus weight percent gain after 300 days of exposure in soil beds. It indicates considerable decrease in weight and MOE_{dyn} losses in acetylated wood. In both species the losses decreased considerably when the degree of acetylation increased. Results showed that at weight gains of about 8% in beech and 10% in pine losses of MOE_{dyn} and mass decreased effectively. Above those WPGs, wood was not attacked considerably. The losses reached almost nil at the highest weigh gains.

The results reveal an inhibitory effect of the acetylation on wood attacking soil microorganisms and show that raising the degree of the acetylation (WPG) reduces microbial decay in the acetylated wood, even when cells are colonized by hyphae (shown below). Results indicate that acetylation above 8% in beech and above 10% in pine begin considerably protect wood from soft rot decay. However, higher WPGs are required for total protection (17% and 19% in beech and pine respectively). These results confirm previous reports. Beckers & Militz (1994) reported that WPG of 10% prevents considerably the soft rot attack on acetylated beech, pine and poplar. Also it has been reported that the acetylation of pine sapwood to a WPG of 10.7% already prevents fungal attack. For poplar a WPG of 14.4% and for beech a WPG of 12.8% was required to achieve the same results (Beckers *et al.*, 1994). Larsson *et al.* (1997) tested the acetylated wood in ground contact and reported that the acetylation has a major impact on the fungal resistance of wood. The resistance of the acetylated wood at WPG of about 20% is at the same range as that of wood with higher retention of preservatives (copper-chromium containing). They also reported that fungal decay in mini-stakes, which were exposed to unsterile soils in laboratory condition were significantly reduced at acetyl content of 15.1%. The acetyl content of 18.5% prevented most attack by tunneling bacteria (Larsson *et al.,* 2000). Takahashi *et al.* (1989) reported an enhancement of decay resistance by acetylation with used

fungi and wood species. Nilsson *et al.* (1988) also tested acetylated particleboards in soil in different cellars and reported that at WPG of about 15%, no attack of microorganisms was observed after 12 month. Ohkoshi *et al.* (1999) reported inhibitory effect of the acetylation at WPG of more than 10% on the fungal degradation.

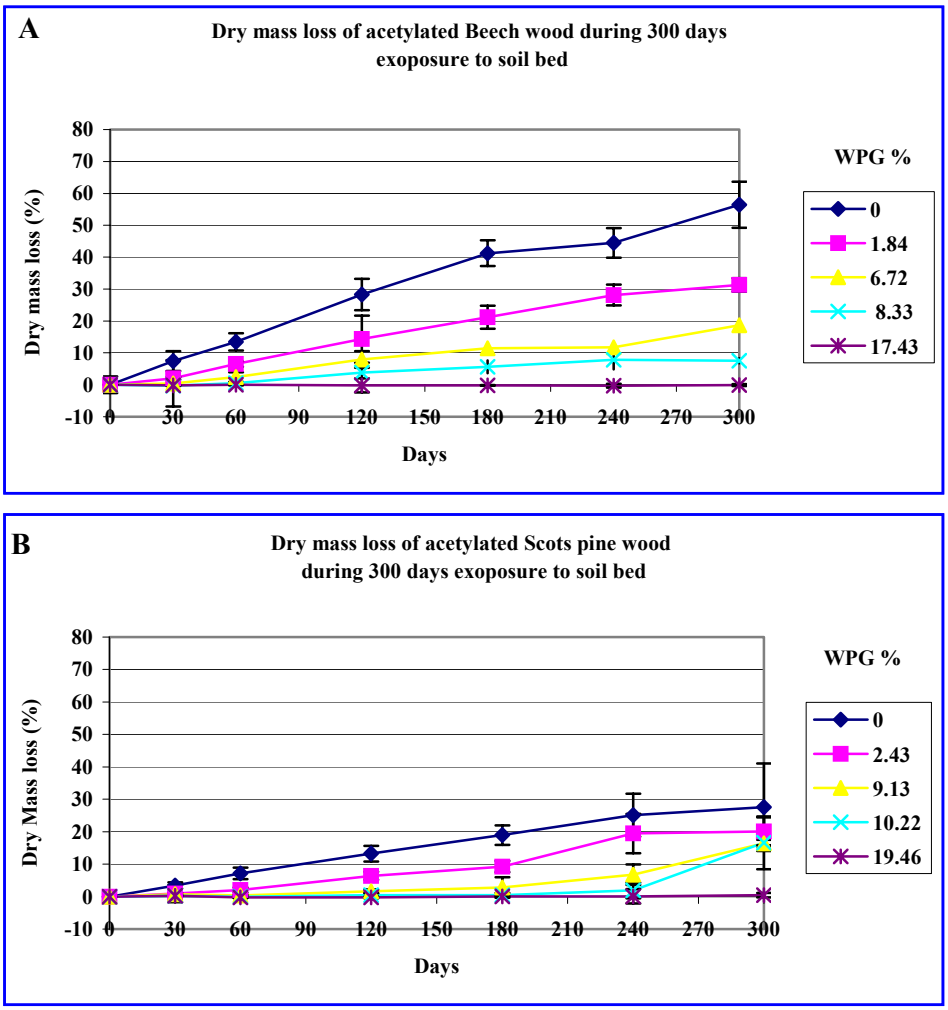


Fig. 2-9. Dry mass losses in acetylated beech (a) and Scots pine (b) wood at different WPGs

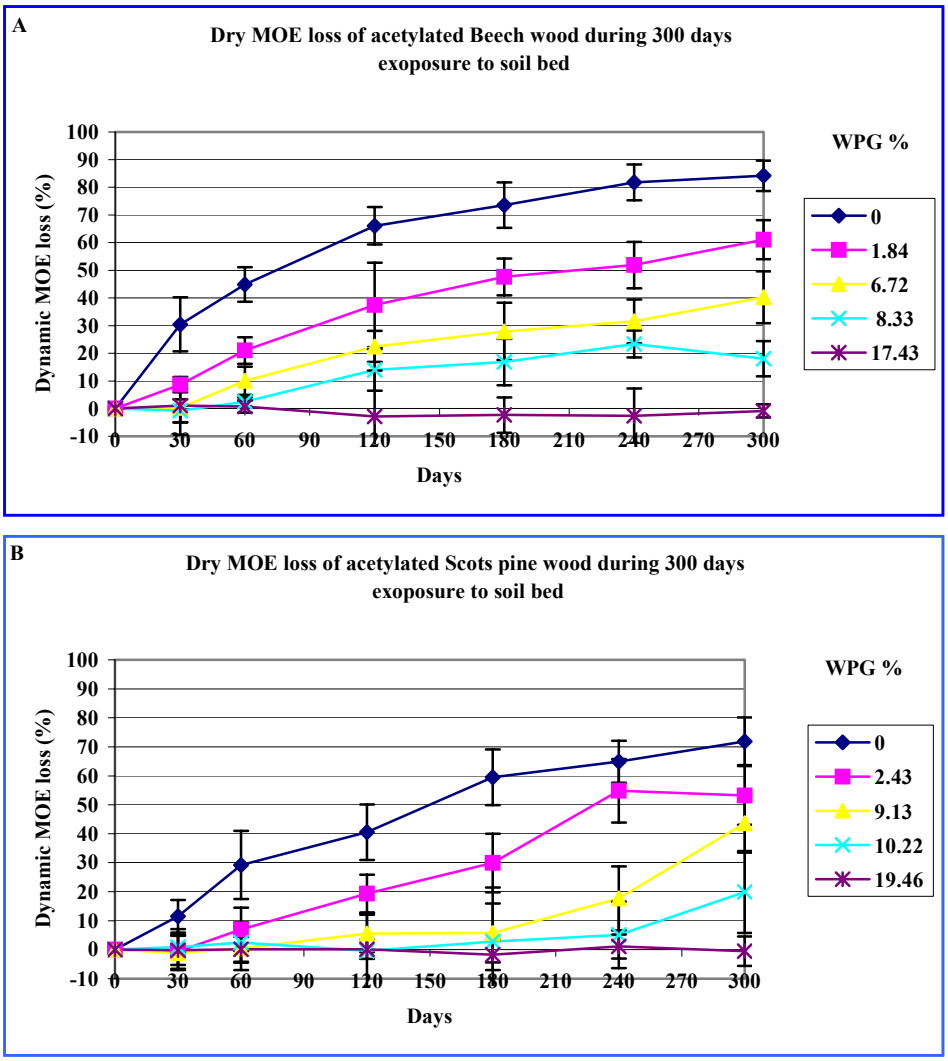


Fig. 2-10. MOE_{dyn} losses in acetylated beech (a) and Scots pine (b) wood at different WPGs

The slight raises in mass and MOE_{dyn} in the highly acetylated woods (figs 2-9 & 2-10) are probably caused due to colonization of hyphae in cell lumina without any attack, which is shown in figure 2-14 I. Because wood mass is influenced by the hyphal mass and any changes in wood mass affects MOE_{dyn}.

Machek *et al.* (1997, 1998a&b, 2001) used vibration technique to determine MOE in attacked wood. They reported that the vibration method is a more sensitive indicator of wood decay than mass loss. Also it has advantage because of time, which could be done during short time and can minimize the period of testing in natural durability in wood. They reported that MOE_{dyn} loses for non-durable and slightly durable species (*e.g.* beech, poplar and elm) ranged from 77-86% and their mass losses after 12 weeks of exposure ranged from 35-40% (Machek *et al.*, 1998a).

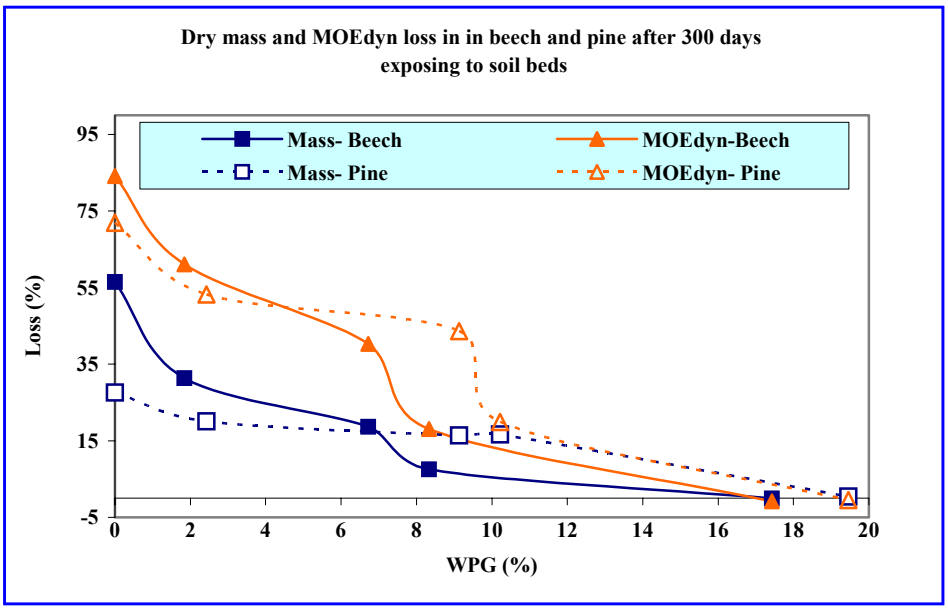


Fig. 2-11. Effect of acetylation on loss of dry mass and MOE_{dyn} after 300 days of exposing to the soil beds

2.4.2. Chemical analysis

Chemical analysis in beech and Scots pine wood showed that loss of cell wall components decreased at raising weight gains after 300 days of exposure to soil beds (figs. 2-12 & 2-13). Main losses were determined in non-acetylated wood and insignificant losses were measured in highly acetylated wood. Concerning the results, it was revealed that acetylation influenced considerably loss of cell wall components and protected wood against soil microorganisms. At weight gains of about 8% in beech and 10% in pine, losses in cell wall components were rapidly decreased (figs. 2-12 & 2-13). At lower than the mentioned weight gains wood loss its components rapidly, while it is very slow and also inhibited in some components at higher than those weight gains. Major losses were occurred in non-acetylated wood. The non-acetylated beech wood lost 64.51% of lignin, 58.45% of holocellulose and 79.28% of α-cellulose based on their initial percentages when wood lost 55.44% of its mass after 300 days of exposure to soil beds. Whereas highly acetylated beech wood at 17.43% of WPG lost only 13.04% of its lignin, while gained 0.45% holocellulose and 9.98% α-cellulose according to their initial percentages when no mass loss was measured (fig. 2-12). Scots pine wood also lost 10.09% of its lignin, 37.46% of holocellulose and 47.82% of α-cellulose based on their initial percentages when wood lost 27.6% of its weight, while in highly acetylated pine wood at 19.46% of WPG, lignin and α-cellulose were increased about 7.59% and 5.59% based on their initial percentage sin wood, while highly acetylated pine wood 4.1% of holocellulose based on the initial percentage (fig. 2-13). Savory and Pinion (1958) reported that beech wood lost its lignin from 19.8% to about 13.5% and α-cellulose from 3.6% to 5.92% in soil test.

Chemical analyses in non-acetylated wood reveal that soft rot fungi could remove cell wall components in different rates. α-Cellulose is removed preferentially, followed by lignin and holocellulose removal in non-acetylated beech wood (fig. 2-12). However, in pine wood α-cellulose is removed initially, followed by removal of holocellulose and lignin (fig. 2-13). Generally, removal of all components of wood is more rapid in beech than in pine. These results are in agreement with Levi and Preston (1965) who suggested that soft rot fungi could remove all wall components in wood.

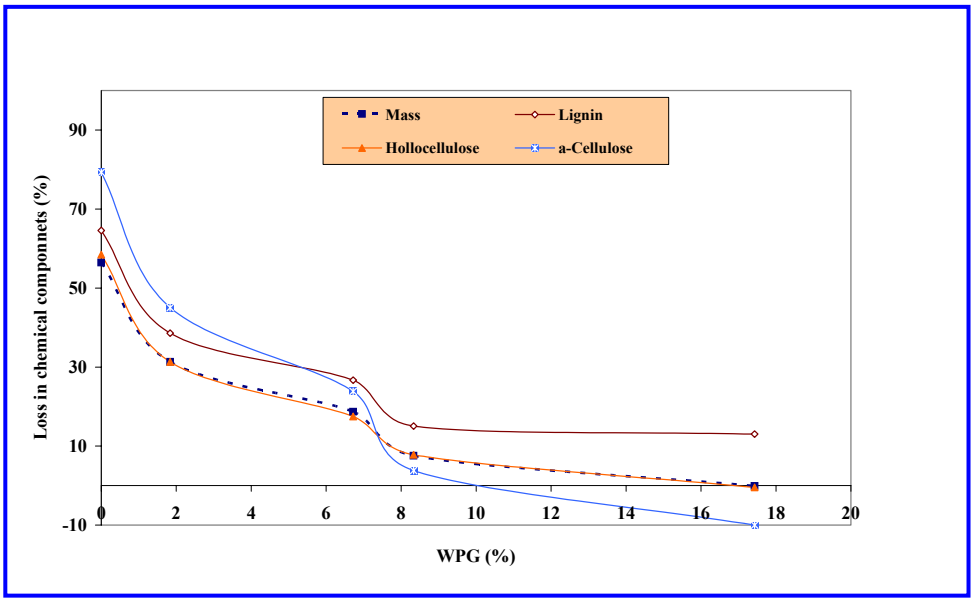


Fig. 2-12. Loss of chemical components in beech based on their initial percentages in wood after 300 days of exposing to soil beds

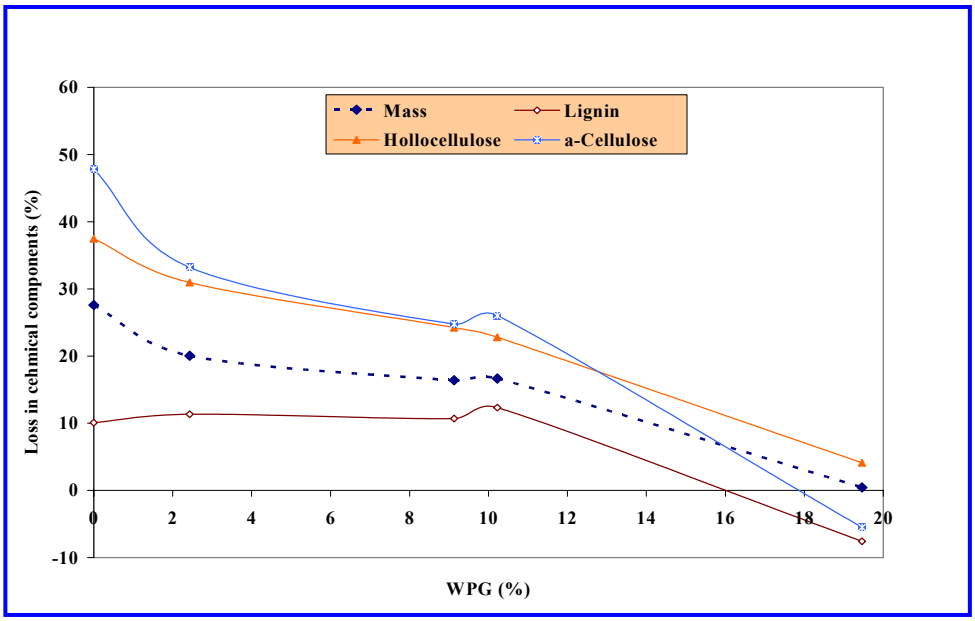


Fig. 2-13. Loss of chemical components in pine based on their initial percentages in wood after 300 days of exposing to soil beds

Chemical analyses showed a significant effect of the acetylation on the removal of chemical components in wood. The removal of all components decreased with raising WPGs in wood. It ceased the removal of α-cellulose at 10% and holocellulose at 17% of weight gains; however it is not able to stop removal of lignin at higher WPGs in beech wood. It seems that a higher WPG is required to cease removal of lignin in beech wood. In pine, removal of α-cellulose stopped at about 18% of weight gain and at ca. 16% for lignin, but holocellulose requires a WPG higher than 20%.

It has been reported that soft rot fungi could remove α-cellulose, hemicelluloses and lignin from wood. However, it was indicated that wood carbohydrates are degraded in preference to lignin (Savory & Pinion, 1958; Levi & Preston, 1965; Seifert, 1966; Nilsson *et al.*, 1989). Levi and Preston (1965) also analyzed removal of Klason lignin and methoxyl groups in lignin by soft rot fungi. Their research showed that the rate of removal for methoxyl groups is considerably higher than Klason lignin itself. Also soft rot fungi can modify lignin during decay. Another study on syringyl : guaiacyl (S:G) ratios of the residual lignin in birch wood decayed by several types of ascomycetous fungi revealed that the syringyl-propane units had been preferentially degraded (Nilsson *et al.*, 1989; Nilsson *et al.*, 1988). As it is also known that lignin of hardwoods have more methoxyl groups and higher S:G ratios than softwoods (Fengel & Wegner, 1989), we can conclude that as lignin in beech has more methoxyl groups and contains higher S:G ratio than the lignin in pine, soft rot fungi decay lignin in beech more preferential than in pine. For this reason, lignin in beech is degraded easier than in pine, even at higher WPGs. Butcher and Nilsson (1982) also demonstrated that high lignin timbers are less prone to soft rot than low lignin timbers. Reports indicated the amount of lignin in beech 22.70% (Butcher and Nilsson, 1982) and Scots pine 27-28% (Nilsson *et al.*, 1988). Our results indicate the amount of Klason lignin for beech 21% and Scots pine 30% (the differences between the reports could be due to different analytical methods). Therefore the higher durability of non-acetylated pine wood in comparison with beech wood probably related to higher lignin content in pine.

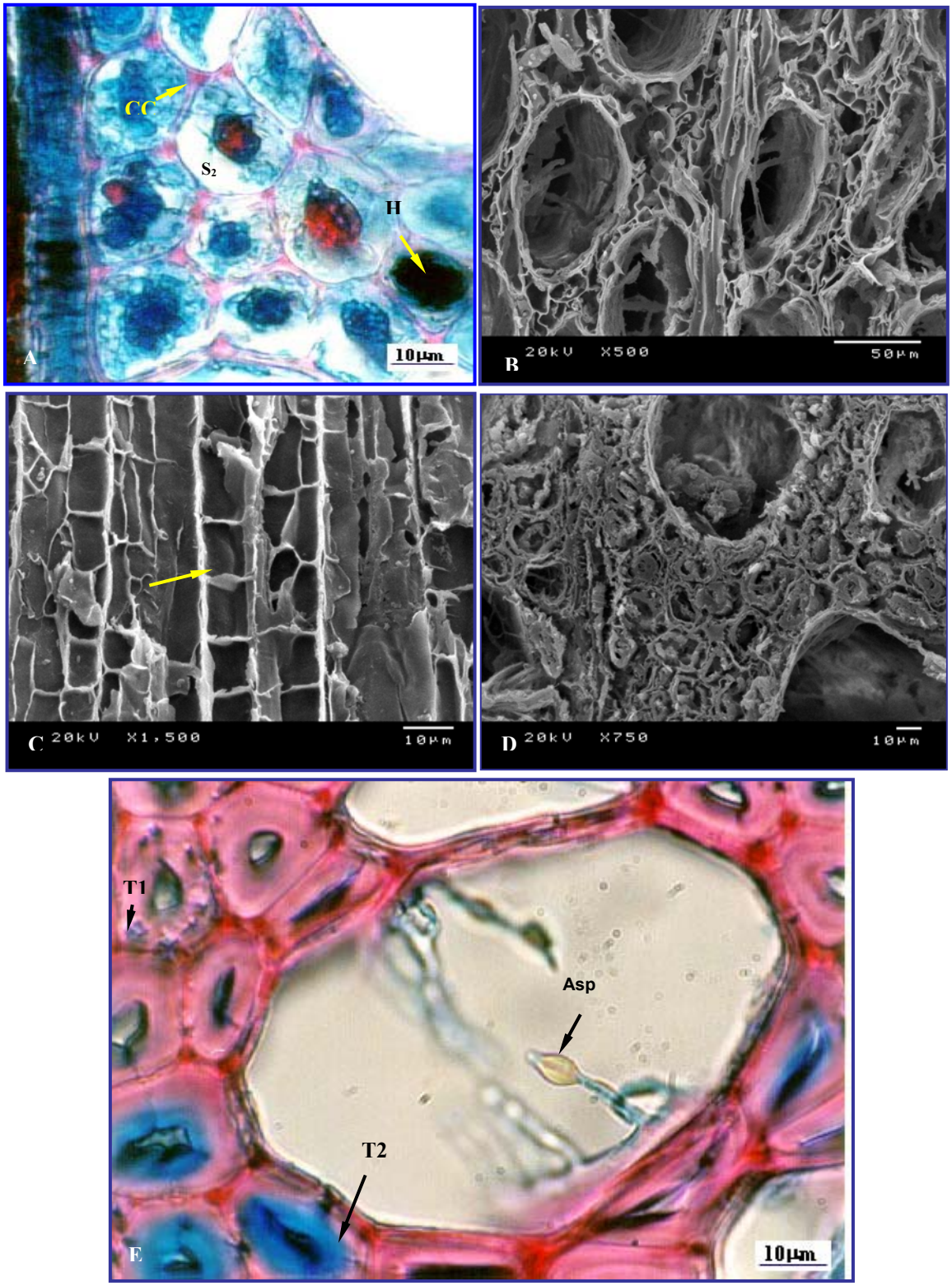
Rosenqvist (2001) reported that uniform distribution of acetyl groups in cell walls occurs at higher weight gains. Ohkoshi *et al.* (1997a&b) studied the acetyl groups in lignin, cellulose and hemicelluloses and reported that substitution of hydroxyl groups in cellulose in amorphous regions begins at WPG 5.7% and all groups could be substituted at WPGs above 20%. However, substitution in all components occurs at higher than 20% of weight gains. From our own results, we can conclude that the differences in removal of cell wall components by soil microorganisms could be due to the differences in degree of substitution of the hydroxyl groups with the acetyl groups in both species during the acetylation, and also differences in chemical structure of the lignin itself (which is more methoxylated in beech than in pine). The hydroxyl groups in the amorphous regions of α-cellulose and hemicellulose could be substituted easier than lignin and cellulose could be rapidly blocked by the acetyl groups at lower WPGs than lignin in beech wood. Levi and Preston (1965) also reported an increase in the average degree of polymerization (DP) of the cellulose in beech wood attacked by *Chaetomium globosum*. This increase suggests a preferential degradation of the amorphous portions of the wood cellulose. Regarding this report, it could be concluded that soft rot fungi has probably no capability to degrade amorphous regions of cellulose due to the acetylation and the acetylation gives a good protection to cellulose against soft rot fungi.

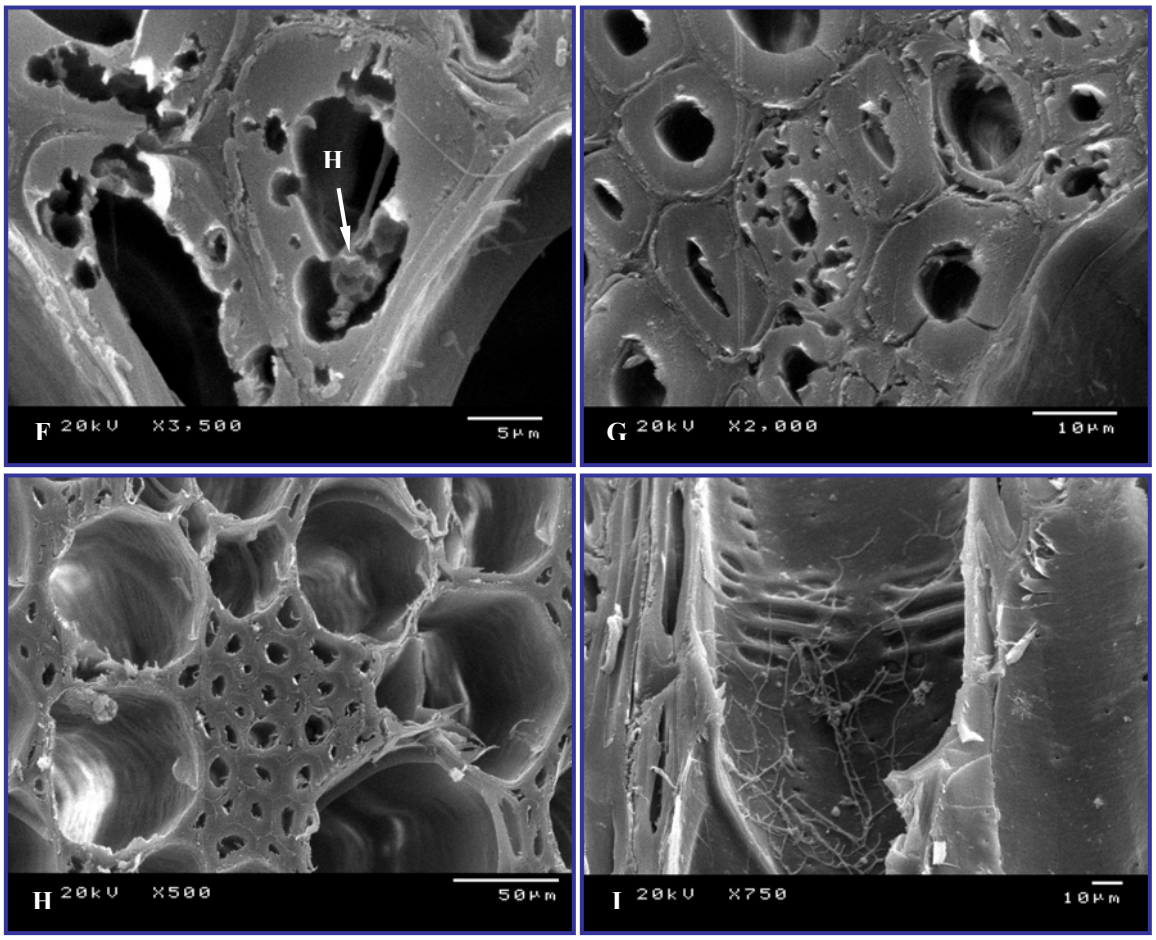
Any raises of chemical components at higher WPGs (in comparison to untreated controls) could possibly be due to the existence of hyphae, which are presented in cell lumina of highly acetylated wood without causing any attack (see fig. 2-14-i). Because, hyphal wall contain polysaccharides and consequently the amount of cellulose in highly acetylated wood is higher than lignin and holocellulose (Burnett & Trinci, 1979).

2.4.3. Microscopy

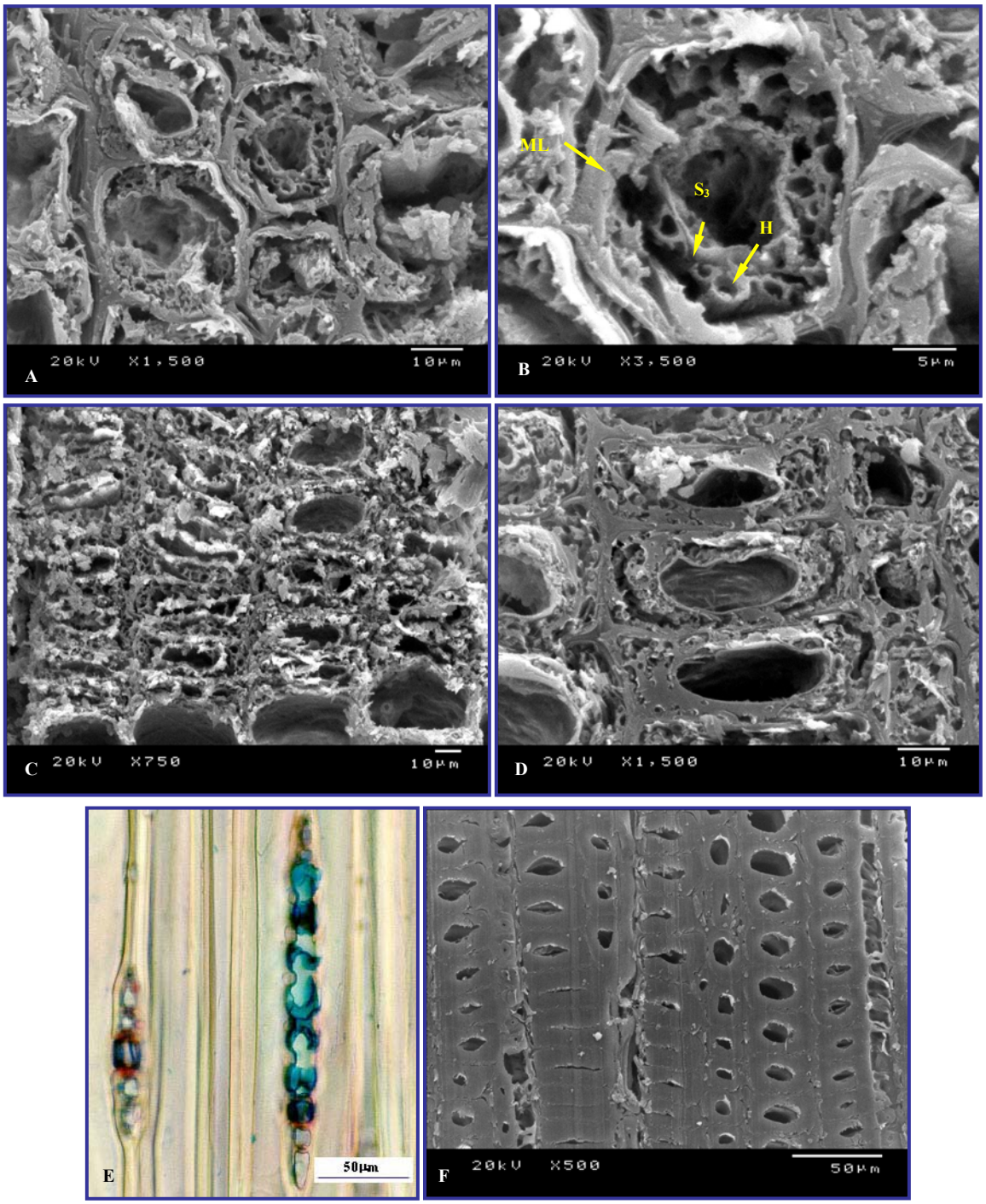
Soft rot decay- Microscopical observations showed a severe attack in non-acetylated and acetylated wood at the lowest WPGs in both species (figs. 2-14&15). Decay patterns were also observed in moderately acetylated woods (figs. 2-14F&G, figs. 2-15D&E). No decay patterns were revealed at the highest WPGs (figs. 2-14H&I, figs. 2-15F). However, fig. 2-14I shows the existence of fungal hyphae in cell lumina. In severely decayed wood, S_2 layer was removed in fiber and tracheid walls; while S_3 layer and ML remained intact (figs. 2-14A-D & figs. 2-15A-D). In those cell walls, hyphae were visible after removal of S_2 layer especially in pine (figs. 2-15A-D). And also in beech fibers, empty cavities were left after removal of cell wall components (fig. 2-14C). These results indicate that soft rot fungi could remove all types of chemical components from S_2 layer. Figure 2-16 shows that soft rot fungi initially modify the cell wall components and then remove them. In this figure hypha is surrounded by modified cell wall components and they are being removed from cell wall. Empty fungal cavities in secondary walls were left after removal of cell wall components. This evidence indicates that soft rot fungi can degrade lignin too. Some authors (Blanchette *et al.*, 1990; Nilsson *et al.*, 1989; Durán *et al.*, 1987; Tanaka *et al.*, 2000) reported the lignolytic activities of soft rot fungi. They have also reported that wood carbohydrates are degraded in preference to lignin. Soft rot fungi have no capability to degrade lignin in compound middle lamella (CML) due to the different chemistry of lignin in CML. It mostly contains guaiacyl (G) type and secondary wall contains syringyl (S)

type. Baeza and Freer (2001) reported the guaiacyl : syringyl ratio in S2 layer in white birch wood fiber 12:88, while it is 91:9 in CML. Reports indicate the capability of soft rot fungi in degrading GS type lignin (Nilsson *et al.*, 1989; Nilsson *et al.*, 1988).





Figs. 2-14- Soft rot decay in beech wood. **A-** Non-acetylated beech, S2 layer is removed, but S_3, ML and cell corners (CC) were remained intact. Hyphae (H) are melanized and fill fiber lumina and rays (dark color). **B&C-** Severe soft rot decay in non-acetylated beech. **c-** S2 layer and its components are removed and empty cavities were remained after attack. This indicates ability of soft rot fungi to remove all chemical components in cell wall. **D&E-** Severe soft rot decay in acetylated beech; WPG 1.84%. **D-** S_2 layer is severely attacked. **e-** Two types of soft rot decay (T_1; typical type I and T_2; erosion type II) occur in neighboring fibers. It indicates soft rot fungi react in different fibers differently. Fruiting body (Asp) of a soft rot hypha has appeared in vessel lumen. **F-** Soft rot decay in moderately acetylated wood; WPG 6.72%. A hypha has penetrated into cell wall and widening its cavity in the S_2 layer by removing its chemical components. **G-** Typical soft rot decay pattern in moderately acetylated beech; WPG 8.33%. **H&I-** No decay in highly acetylated beech; WPG 17.43%. **I-** Cell walls are not decayed even hyphae colonized in the cell lumina.



Figs. 2-15- Soft rot decay in pine wood. **A&B-** Severe soft rot decay in non-acetylated pine. S_3 layer and ML are remained intact; while S_2 layer is totally removed by hyphae. The hyphae in S_2 layer are visible in cross view as rounded. **C-** Severe soft rot decay in acetylated pine; WPG 2.43%. S_3 layer and ML are seen intact. Also round shape hyphae are visible inside the S2 layer. **D-** Soft rot decay in acetylated pine; WPG 9.13%. Typical soft rot patterns are seen in S_2 layer. S_3 layers and ML are visible intact. **E-** Soft rot fungi decay rays easily in moderately acetylated pine; WPG 10.46. **F-** No decay in highly acetylated pine; WPG 19.46%. Tracheid cell walls are bulked and their lumina became smaller due to acetylation.

In beech wood, two types of soft rot decay patterns, *typical type-I* (produce cavities in S_2 layer) and *erosion type-II* (erode cell walls) (Anagnost, 1998; Blanchette *et al.*, 1990; Kim & Singh, 2000; Daniel & Nilsson, 1998), were observed in neighboring fibers (fig. 2-14 E). It has been reported that soft rot fungi type-I produce cavities in secondary wall and type-II erode cell wall through lumina. However the later one is active in hardwood and low lignin content species (Singh & Kim, 1997; Blanchette *et al.*, 1990; Kim & Singh, 2000). Beech wood contains less lignin comparing Scots pine. Fruiting bodies of growing soft rot hyphae were also observed in vessel lumina (fig. 2-14E). It indicates a suitable condition for hyphal growth in the wood.

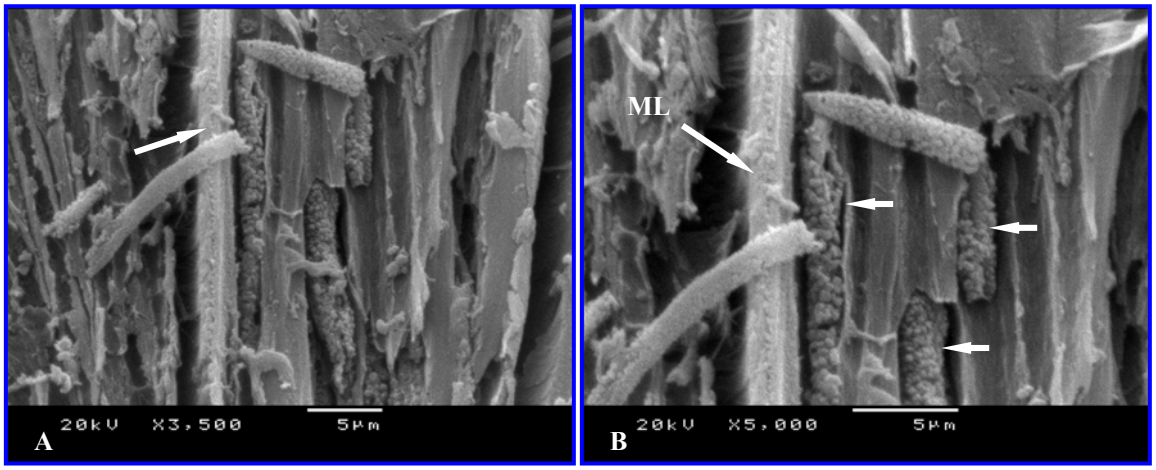


Fig. 2-16. Hyphae modify cell wall components and alter them into granular shapes (mottled erosion) before use within the cell walls (arrow heads): severely decayed acetylated pine wood: WPG 9.13%. ML: Middle Lamella

In moderately acetylated beech fiber, it was observed that a penetrated hypha is widening the hyphal tunnel in the S_2 layer by removing its whole components (fig. 2-14F).

Empty cavities in S_2 layer, which were observed under microscope, prove that soft rot fungi can remove all types of chemical components, but are not able to remove the highly lignified compound middle lamella and S_3 layer. Donaldson (1987) reported a lignin content of 53% for S_3 layer, 22% for S_2 layer and 86% for cell wall corner compound middle lamella. Because compound middle lamella is rich in lignin than the secondary wall where contains lower concentration of lignin (Fengel & Wegner, 1989; Gindl, 2001; Savory & Pinion, 1958). Due to high concentration of lignin, fungal hyphae cannot degrade ML. Also type of lignin differs between secondary wall and ML. Secondary wall contains mostly syringyl type of lignin, while ML is rich in mostly guaiacyl type (Baeza & Freer, 2001).

Different authors reported inhibitory effect of the acetylation on fungal growth (Nilsson and Daniel, 1989; Schmitt *et al*, 1996; Fitzgerald & Line, 1990). They suggested that the enzymes required for denaturation of lignin are found more commonly associated with basidiomycetous than deutromycetous fungi. Dark hyphae in cell lumina and ray cells, which were found in our microscopical studies, are due to their melanized structure (Nilsson *et al.*, 1989).

Soft rot decay in rays of beech wood was also observed in all WPGs except the highest WPG (17.43%) (fig. 2-17). It shows that rays are severely decayed especially in the non-acetylated beech wood (fig. 2-17A). Secondary wall is also severely decomposed by hyphae, while ML is still remaining intact.

It is well known that the rays are the best pathways for microorganisms to penetrate into the wood. It can be concluded that hyphae have decayed the rays to make enough spaces to colonize during the first days of wood decay and also use the reserved nutrients in the rays, because they are as storage of metabolites that contain useful nutrients for microorganisms.

Microscopical observations confirm that raising WPG by the acetylation processes prevents biological attack in the acetylated beech and pine wood and also it ceases the decay at higher WPGs.

Our observations also confirm findings of Kumar (1994) that decay hyphae limit themselves to parenchyma cells and tracheid lumina and fail to attack the acetylated cell walls.

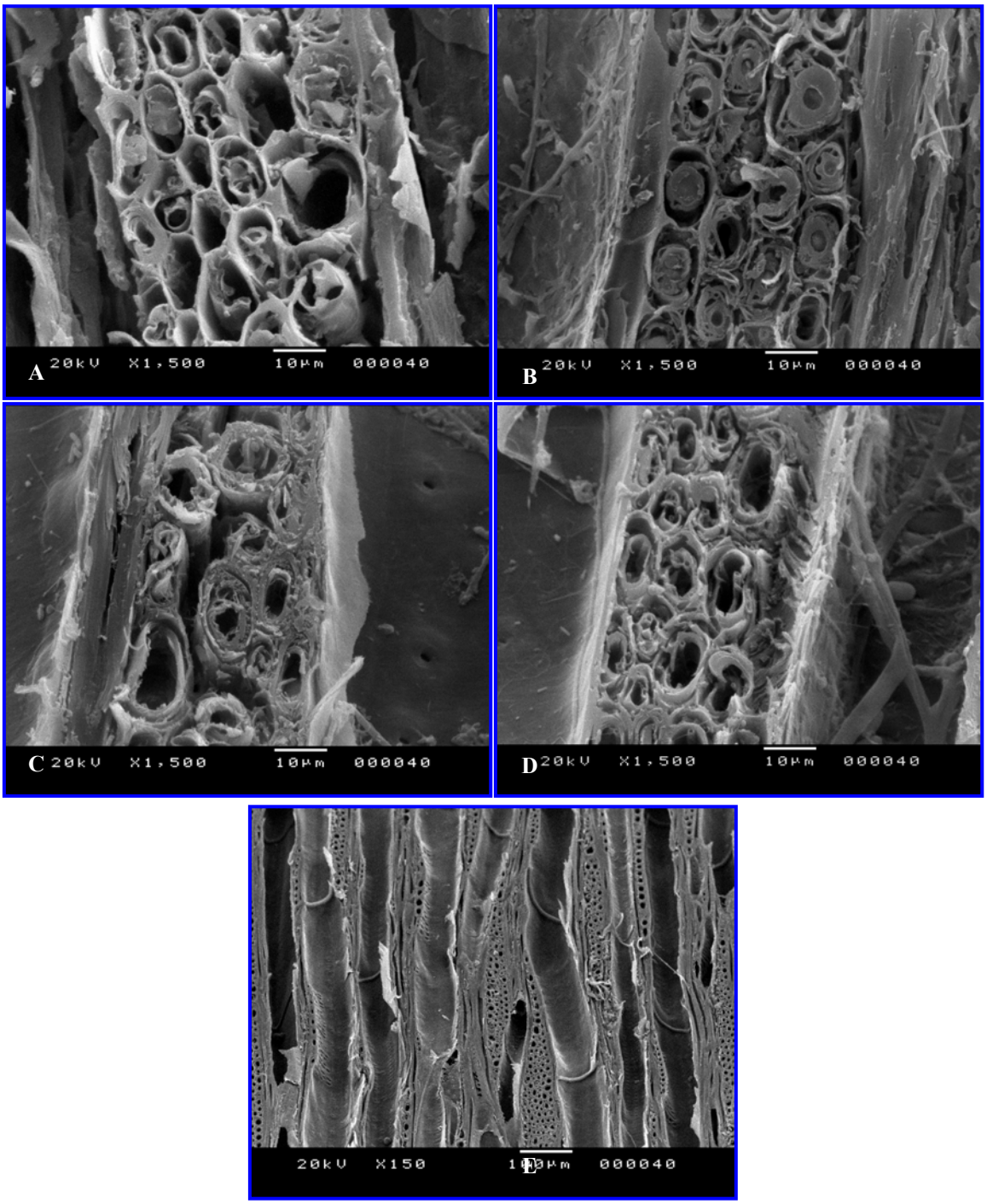
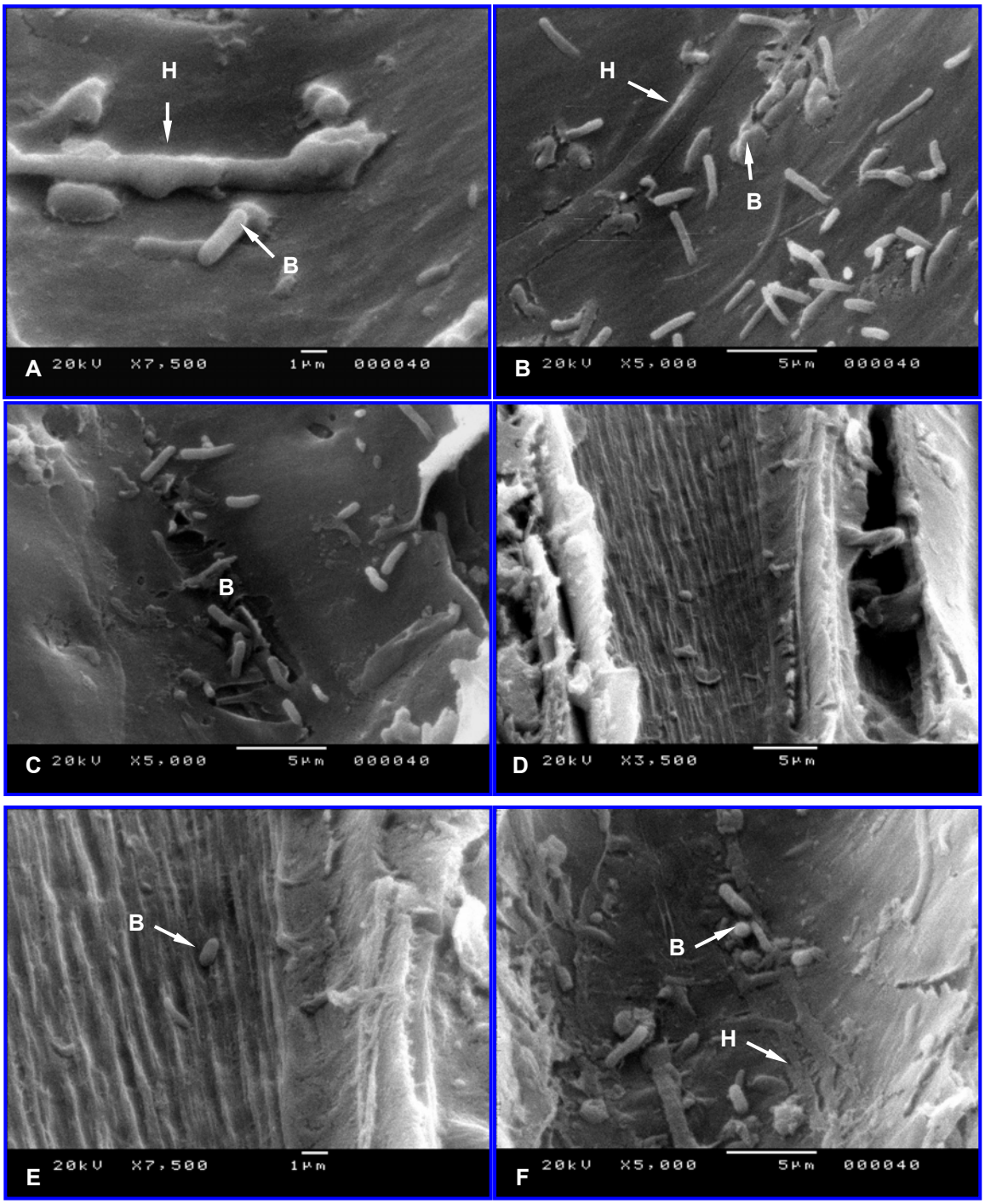


Figure 2-17. Soft rot decay in ray cells of beech wood after a long period of exposing to soil microorganisms. **A:** non-acetylated beech. **B:** acetylated beech; WPG 1.84%. **C:** acetylated beech; WPG 6.72%. **D:** acetylated beech; WPG 8.33%. **E:** acetylated beech; WPG 17.43%.


H
B
A 20kV X7,500 1µm 000040
H
B
B 20kV X5,000 5µm 000040
B
C 20kV X5,000 5µm 000040
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B
H
F 20kV X5,000 5µm 000040

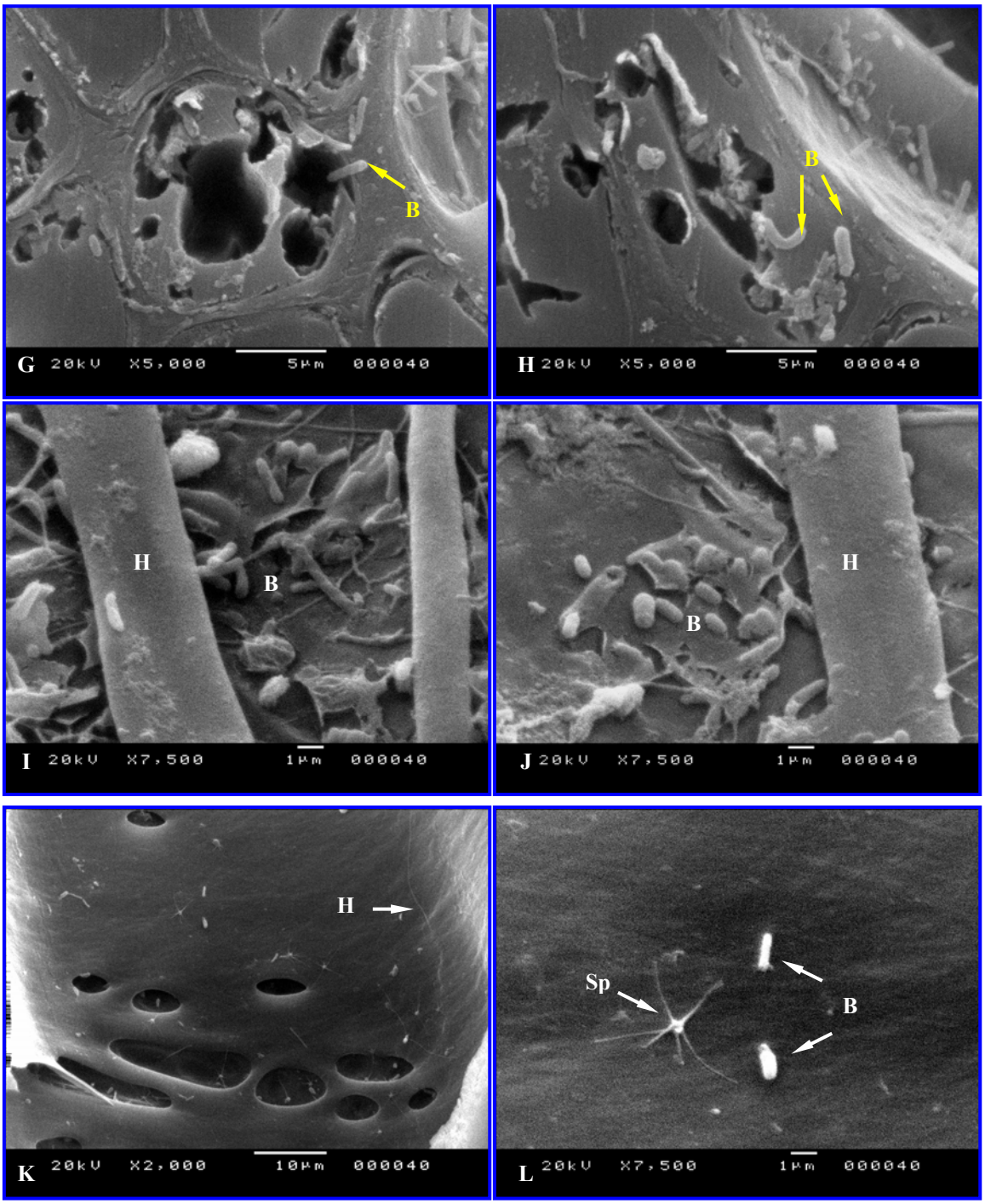

Figure 2-18- Bacteria and bacterial decay in non-acetylated and acetylated beech wood. **A-D:** Non-acetylated beech wood. **a:** Bacteria are very close to soft rot hyphae. **B:** The closest bacteria to hyphae are sinking into cell wall. **c:** Bacteria are following the trace of a penetrated soft rot hypha into cell wall. **D&E:** Erosion bacterial decay on cell wall. **F:** Fungal hyphae are surrounded by bacteria: WPG 1.84%. **J&H:** Bacteria existing in soft rot hyphal tunnels: WPG 6.72%. **I&J:** Number of bacteria around fungal hyphae: WPG 8.33%. **K&L:** There is no decay in highly acetylated beech wood. But there exist very thin hyphae in cell lumina.

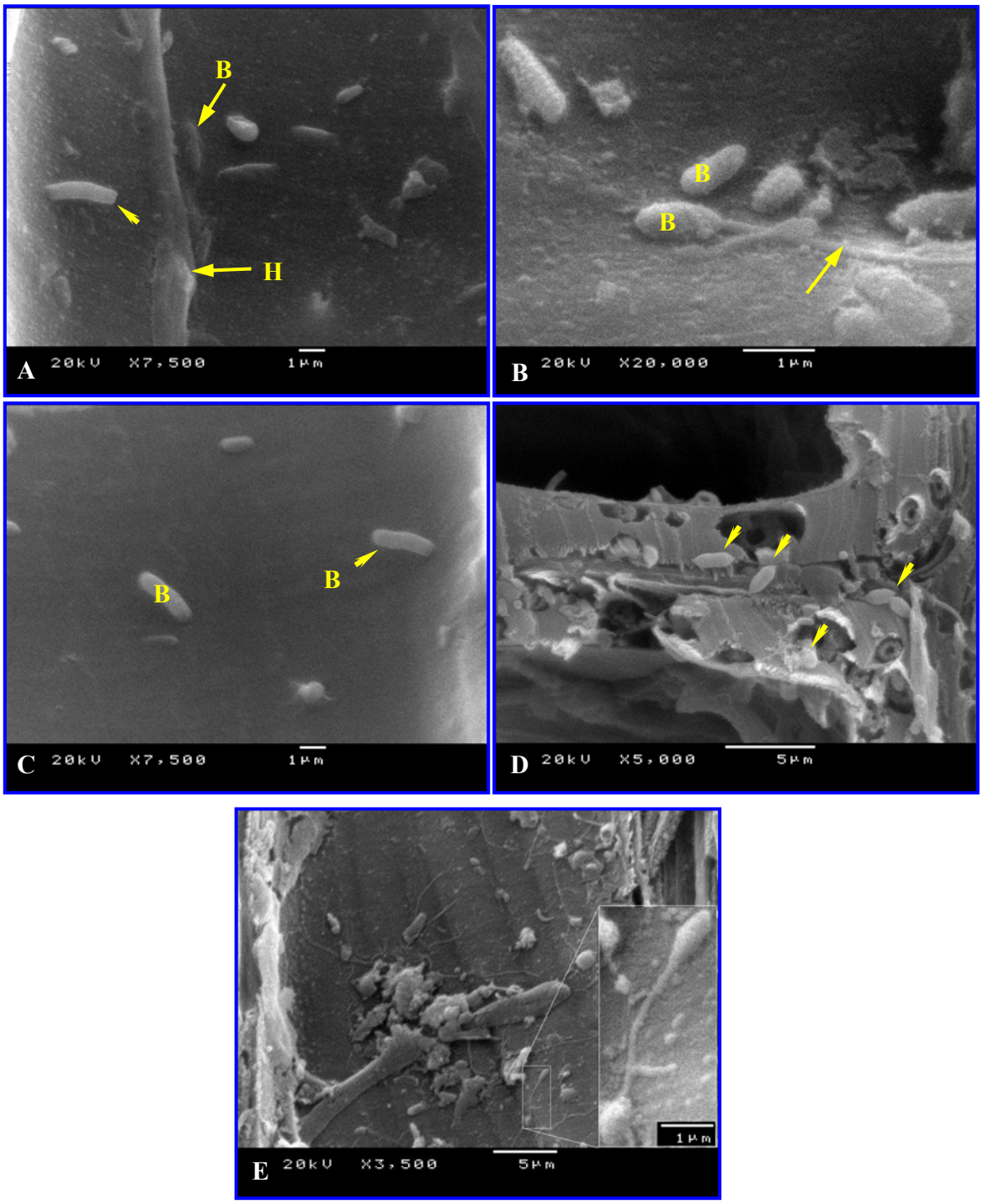

Fig. 2-19. Bacteria (B) and bacterial decay in Scots pine wood. **A&B:** Non-acetylated pine wood. Bacteria are following the hypha (H) and sinking into the cell wall (arrows). Also a bacterium is penetrating into the cell wall to begin the decay it (arrow head). **C-E** Acetylated pine wood. **C:** Penetration of bacteria into the cell wall (arrow heads): WPG 2.43%. **D:** Bacteria in cell wall and hyphal tunnels: WPG 9.13%. **E:** Bacteria surrounding the hypha. Flagellate bacteria also exist in the cell wall: WPG 9.13%.

Bacterial Decay- Figure 2-18 shows presence of bacteria and also bacterial decay in non-acetylated and acetylated beech wood. Erosion bacterial decay was distinguished in non-acetylated beech (fig. 2-18D-E). It was also observed that enormous bacteria were closely surrounding fungal hyphae in non-acetylated (fig. 2-18A-C) and acetylated beech (fig. 2-18F-J).

In non-acetylated beech, not only bacteria are in close contact with fungal hyphae (fig. 2-18A&B), but also follow traces of deeply sunk fungal hyphae in cell wall (fig. 2-18C). Bacteria were also found near to hyphae in low levelly acetylated beech (fig. 2-18F) and also in fungal tunnels (fig. 2-18-g&h). They were observed in fungal decayed cell walls of the acetylated wood (fig. 2-18I&J), although no decay was observed at the highest WPG in the acetylated beech wood. However there were very thin hyphae that were surrounded closely by bacteria (fig. 2-18K). Also the presence of bacteria was distinguished very near to fungal spores (fig. 2-18-l).

In non-acetylated pine wood, bacteria were also observed around hyphae (figs. 2-19A&B). Penetration of bacteria into the cell walls was observed in the non-acetylated and the acetylated pine wood (figs 2-19A&C). In the acetylated woods presence of the bacteria in the cell walls were also revealed (figs 2-19D&E). Bacteria were observed in two shapes of "flagellate" and "rod shape". Flagellate bacteria are motile bacteria and produce tunneling degradation in the cell walls (Daniel & Nilsson, 1998). No bacterial degradation was observed in the highly acetylated wood.

The presence of bacteria around hyphae indicates a close association between bacteria and fungi to decay wood. It can be a synergism between them to ease decay in wood. Bacteria can act as nitrogen fixers for fungi during decay process. Probably flagellate bacteria, which are observed in pine wood, are responsible for a tunneling type of degradation in the wood. Nilsson *et al.* (1988) have reported that tunneling bacteria can degrade acetylated wood. However no bacterial decay was observed in the highly acetylated woods of both species. Absence of bacteria or bacterial decay indicates good protection of acetylation at higher weight gains against bacteria.

2.4.4. IR spectra of acetylated wood

Acetylation causes some significant changes in IR spectra of wood (fig. 20&23). The hydroxyl groups (O-H) diminish at about 3354-3328cm^{-1} due to substitution of the hydroxyl groups in lignin, polysaccharides (Sundell *et al.*, 2000; Faix 1991, 1996; Fengel & Wegner, 1980; Kajihara *et al.*, 1993; Pandey, 1998; Pandey & Theagarjan, 1997), because the substitution of hydroxyl groups with acetyl groups prohibits absorption of moisture from environment. A strong peak appears at about 1733-1728cm^{-1} in beech and 1737-1728cm^{-1} in Scots pine due to the carbonyl (C=O) stretching in carboxyl groups. The magnitude of this band increases with raising the weight gains (Faix 1991, 1996; Zhang & Kamdem, 2000; Stewart & Morrison, 1992; Pandey & Theagarjan, 1997; Sundell *et al.*, 2000; Takahshi *et al.*, 1992; Evans *et al.*, 1992). This peak is related to substitution of the hydroxyl groups in polysaccharides with the acetyl groups. The methyl deformation of the acetyl groups induces an increase at about 1369cm^{-1} due to the stretching of C-H in both acetylated wood's polysaccharides (Evans *et al.*, 1992; Sundell *et al.*, 2001). There is also a clear increase in the intensity at about 1255-1226cm^{-1} in beech and 1261-1226cm^{-1} in Scots pine due to the stretching of C-O and carbonyl deformation in the ester bonds during the acetylation (Sundell *et al.*, 2000; Takahashi *et al.*, 1989; Sundell *et al.*, 2001; Faix & Böttcher, 1993). This peak also related to lignin.

<u>IR spectra in decayed beech wood</u>- Figure 2-20 shows the IR spectra in acetylated and non-acetylated beech wood before and after 300 days of soil exposure. The assignments could be explained as below:

a- Wave numbers 1737-1728 cm^{-1} represent C=O stretching in acetyl in hemicelluloses (Sundell *et al*, 2000; Stewart & Morrison, 1992; Faix, 1991, 1996; Zhang & Kamdem, 2000; Kimura *et al.*, 1992; Pandey & Theagarjan, 1997; Takahashi *et al.*, 1989; Evans *et al.*, 1992; Kosikova & Bucko, 1998).

C=O stretching of the acetyl groups in hemicelluloses was decreased at the increased degree of acetylation. The decrease probably related to cleavage of the acetyl groups in hemicellulose; especially xylan by esterases.

b- Wave number 1595 cm^{-1} represents OH stretching of linked water to cellulose (Fengel & Wegner, 1980).

OH groups increase in all types of wood. The increase in hydroxyl groups probably related to attacking glycosidic linkages in polysaccharides by hydrolyzing enzymes (cellulases). Acetylation does not influence these linkages.

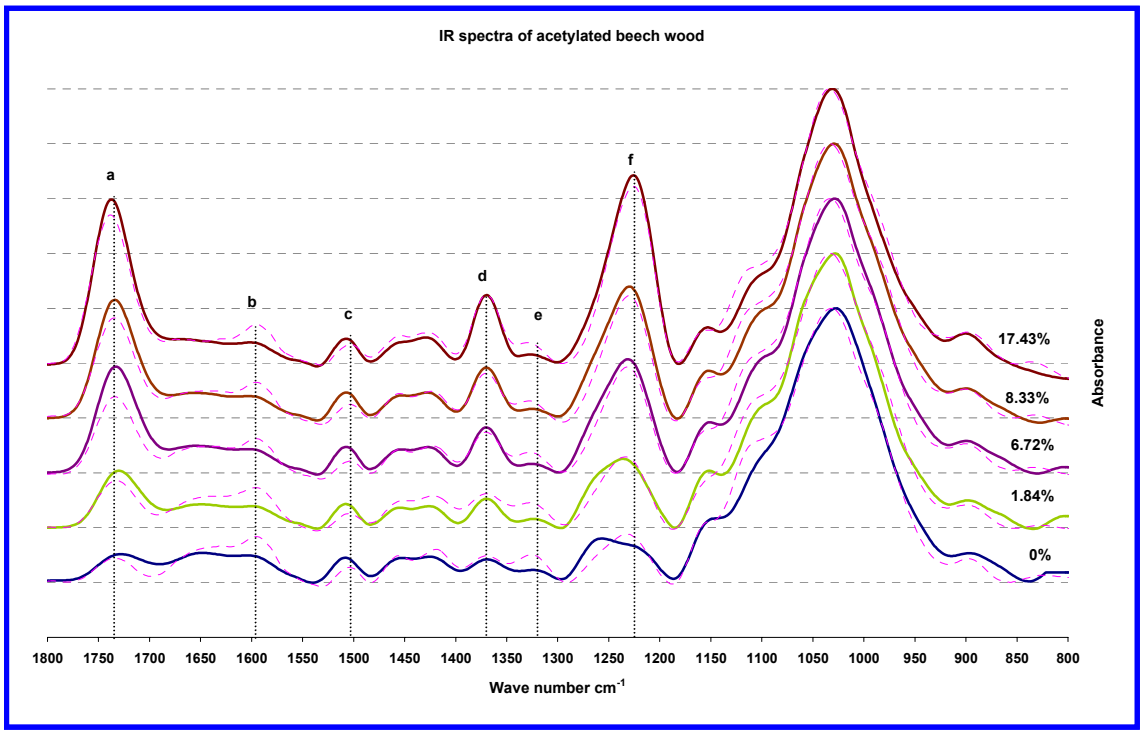

Fig. 2-20- IR spectra of acetylated beech wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil. Wave numbers:

a: 1737-1728cm^{-1}; b: 1595cm^{-1}; c: 1506-1502cm^{-1}; d: 1370-1369cm^{-1}; e: 1330-1325cm^{-1}; f: 1234-1226cm^{-1}

c- Wave numbers 1506-1502 cm^{-1} represent C=C stretching vibration in aromatic ring in lignin or/ aromatic skeletal in lignin (Kimura *et al.*, 1992; Evans *et al.*, 1992; Rodrigues 1998; Pandey & Theagarjan, 1997; Schultz & Glasser, 1986; Pandey, 1998).

Reduction in the aromatic skeletal vibration explains that microorganisms decay lignin. Except the highest WPG, decrease in the skeletal vibration occurs in the non-acetylated wood, the low and the moderately acetylated woods.

d- Wave numbers 1370-1369 cm^{-1} represent C-H deformation in CH_3 from the acetyl groups due to the acetylation in lignin and OH groups from aromatic ring (Sundell *et al.*, 2000; Schultz & Glasser, 1986; Zhang & Kamdem, 2000; Pandey & Theagarjan, 1987; Evans *et al.*, 1992).

Increase in OH groups from aromatic ring in the non-acetylated wood and the lowest level of the acetylation probably refers to cleavage of ether bonds in lignin (e.g. β-O-4) by lignolytic enzymes, which were released sites for OH groups to be bonded with aromatic ring. The reduction of this band in the moderately acetylated woods (6.72 and 8.33%) indicates a deacetylation process by de-esterase enzymes. No changes have been occurred at the highest WPG.

Lignolytic activities of soft rot fungi have been reported (Levi & Preston, 1965; Blanchette *et al.*, 1990; Nilsson *et al.*, 1989; Durán *et al.*, 1987; Tanaka *et al.*, 2000).

e- Wave numbers 1330-1325 cm^{-1} represent C-O of syringyl ring and OH of primary and secondary alcohols and also syringyl lignin breathing (Schultz & Glasser, 1986; Faix, 1991, 1996; Fengel & Wegner, 1980).

Increase of C-O in syringyl lignin and OH groups from primary and secondary alcohols in all non-acetylated and acetylated woods probably indicate cleavage of syringyl lignin at C_1-C_α, where primary alcohol is produced on $C\alpha$ from aliphatic propane chain in lignin and also C-O bond from OH group on C_1 from syringyl ring. This indicates that lignolytic enzymes react on C_1-C_α in lignin of acetylated and non-acetylated lignin.

f- Wave numbers 1234-1226 cm^{-1} represent C=O deformation in ester bonds formed during the acetylation and C-O stretching in syringyl lignin (Faix & Böttcher, 1993; Kimura *et al.*, 1992; Schultz & Glasser, 1986; Faix, 1991, 1996; Zhang & Kamdem, 2000; Sundell *et al.*, 2001).

C=O stretching was reduced in the acetylated wood due to the deacetylation process, while C-O stretching in syringyl lignin was increased due to the cleavage of β-O-4 links.

IR spectra in decayed beech lignin- Figure 2-21 reveals IR assignments of Klason lignin in the acetylated and the non-acetylated beech wood before and after 300 days exposing to the soil. The assignments are indicated in the graph.

Klason lignin preparation is a harsh method, because sulfuric acid 72% is used to dissolve polysaccharides and precipitate lignin. The chemical structure of the lignin is modified and oxidized by the acid. Therefore, there are many changes that are not known whether they are due to the lignin preparation or soil microorganisms. For example, the prominent peaks of the acetylation are disappeared due to deacetylation (wave numbers 1737-1728 cm^{-1} and 1234-1226 cm^{-1}) during lignin preparation and also aromatic skeletal vibrations are probably reduced due to strong oxidation by sulfuric acid.

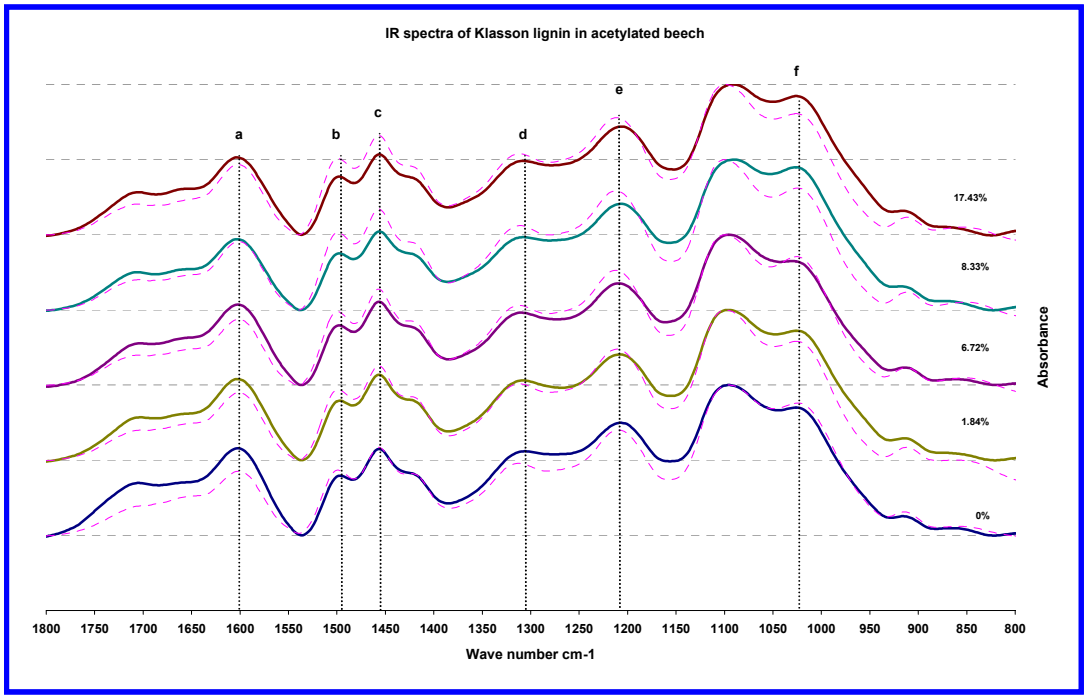

Fig. 2-21- IR spectra of Klason lignin in acetylated beech wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil. Wave numbers:

a: 1602cm^{-1}; b: 1510-1496cm^{-1}; c: 1460-1456cm^{-1}; d: 1329-1315cm^{-1}; e: 1209-1207cm^{-1}; f: 1030-1026cm^{-1}

IR spectra in decayed beech cellulose- Figure 2-22 indicates alteration of cellulose in the acetylated and the non-acetylated wood before and after 300 days of exposure to the soil microorganisms.

Cellulose preparation is also a harsh method that can modify its structure by strong alkaline (NaOH 17.5%). No considerable difference between cellulose of the acetylated and not-acetylation wood, which has shown in figure 2-22, indicates that the chemical structure of cellulose could be affected by alkaline. For example, the prominent peaks for the acetylation were disappeared, while, they are still remaining in the tested wood samples.

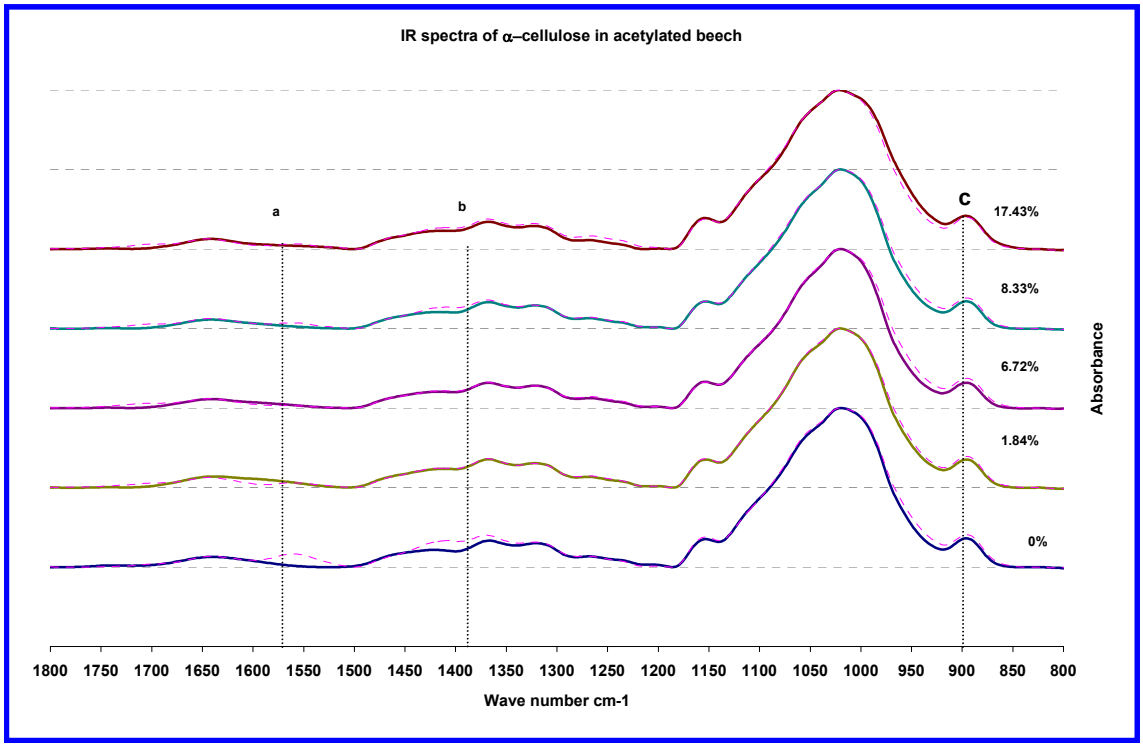

Fig. 2-22- IR spectra of α-cellulose in acetylated beech wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil. Wave numbers:

a: 1602cm^{-1}; b: 1510-1496cm^{-1}; c: 1460-1456cm^{-1}; d: 1329-1315cm^{-1}; e: 1209-1207cm^{-1}; f: 1030-1026cm^{-1}

IR spectra in decayed Scots pine wood- Figure 2-23 shows the IR spectra in acetylated and non-acetylated pine wood before and after 300 days exposing to the soil beds. The assignments are explained as below:

a- Wave numbers 1737-1730cm^{-1} represent C=O stretching in the acetyl groups in hemicelluloses increased due to the acetylation (Sundell *et al*, 2000; Stewart & Morrison, 1992; Faix, 1991, 1996; Zhang & Kamdem, 2000; Kimura *et al.*, 1992; Pandey & Theagarjan, 1997; Takahashi *et al.*, 1989; Evan *et al.*, 1992; Kosikova & Bucko, 1998).

C=O stretching of the acetyl groups in hemicelluloses was decreased at the increased degree of acetylation. The decrease probably related to cleavage of the acetyl groups in hemicellulose; especially xylan by esterases.

b- Wave numbers 1666-1643cm^{-1} represent OH stretching of linked water to cellulose (Fengel &Wegner, 1980; Evans *et al.*, 1992).

OH stretching increases at the lowest WPG and the moderately acetylated wood. No increase was measured at the highest WPG. Due to the deacetylation in the polysaccharides, OH groups could substitute free sites. Less increase in the non-acetylated wood is related to its low content of acetyl groups.

c- Wave numbers 1236-1224cm^{-1} represent C-O of guaiacyl ring and C=O stretching in ester bonds due to the acetylation (Faix & Böttcher, 1993; Kimura *et al.*, 1992; Schultz & Glasser, 1986; Faix, 1991, 1996; Zhang & Kamdem, 2000; Sundell *et al.*, 2001; Collier *et al.*, 1992).

Reduction in C=O stretching occurs at the lowest WPG and the moderately acetylated wood. No change occurs at the highest WPG. The reduction of C=O stretching is related to the deacetylation process which occurs by esterase enzymes. Any reduction in C-O of guaiacyl rings in the non-acetylated wood could be related to the demethoxylation.

d- Wave numbers 1143-1141cm^{-1} represent aromatic C-H plane deformation, typical for G units (Faix, 1991, 1996; Faix & Beinhoff, 1988; Sundell *et al.*, 2000; Pandey, 1998).

The aromatic C-H deformation was increased in the non-acetylated wood and at the lowest WPG. Slight increase was also measured in the moderately acetylated wood. No change occurs at the highest WPG. Any raise in C-H deformation of aromatic guaiacyl ring could be related to the demethoxylation. The demethoxylation by *Chaetomium globosum* was reported in wood (Levi & Preston, 1965).

The main changes in the acetylated wood except the highest WPG, is the deacetylation process that occurs in the carbohydrates and lignin. Due to the deacetylation in wood, free sites in carbohydrates by OH groups from wet soil environment. The demethoxylation in guaiacyl lignin rings except the highest WPG proves well protection of lignin due to the acetylation. No alteration at the highest WPG proves well protection of wood due to the acetylation.

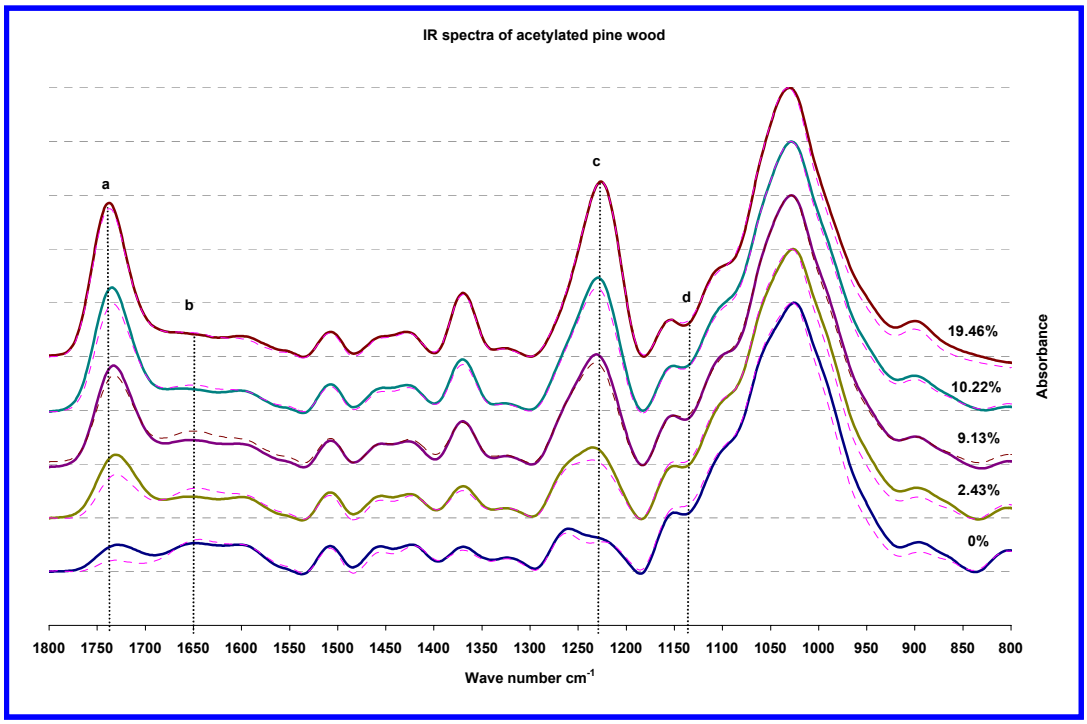

Fig. 2-23- IR spectra of acetylated Scots pine wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil. Wave numbers:

a: 1737-1730cm^{-1}; b: 1666-1643cm^{-1}; c: 1236-1224cm^{-1}; d: 1143-1141cm^{-1}

IR spectra in decayed Scots pine lignin- Figure 2-24 reveals IR assignments of Klason lignin in the acetylated and the non-acetylated beech wood before and after 300 days exposing to the soil. The assignments are indicated in the graph.

As explained above Klason lignin preparation is a harsh method, because sulfuric acid 72% is used to dissolve polysaccharides and precipitate lignin. The chemical structure of the lignin is modified and oxidized by the acid. Therefore, there could be many changes that are not known whether they are due to the lignin preparation or soil microorganisms. For example, the prominent peaks of the acetylation are disappeared due to deacetylation (wave numbers 1737-1728 cm^{-1} and 1234-1226 cm^{-1}) during the lignin preparation and also aromatic skeletal vibrations are probably reduced due to strong oxidation by sulfuric acid.

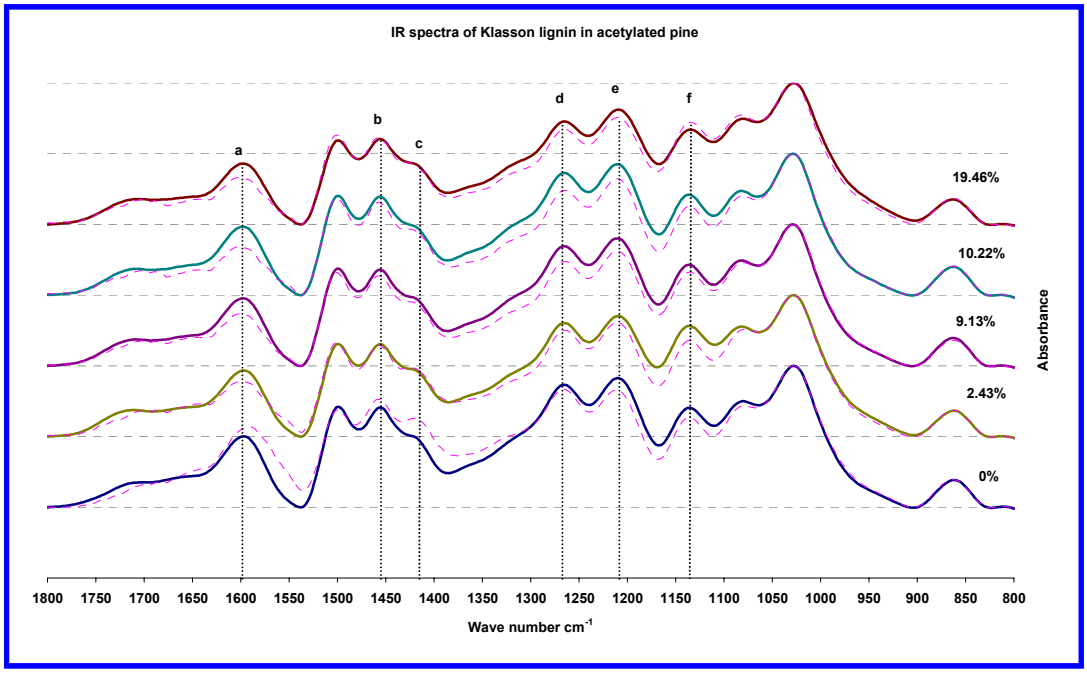

Fig. 2-24- IR spectra of Klason lignin in acetylated Scots pine wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil: Wave numbers.

a: 1602-1591cm^{-1}; b: 1458cm^{-1}; c: 1421cm^{-1}; d: 1265cm^{-1}; e: 1211-1209cm^{-1}; f: 1136-1134cm^{-1}

IR spectra in decayed Scots pine cellulose- Figure 2-25 indicates the IR spectra of cellulose in the acetylated and the non-acetylated pine before and after 300 days of exposing to the soil. The preparation of cellulose is also a harsh method and the chemical structure of cellulose could be altered during the preparation by NaOH (17.5%). However, the assignments could be explained as below:

a- Wave numbers 1600-1590 cm^{-1} represent OH stretching linked water to cellulose (Fengel & Wegner, 1980).

OH stretching of linked water to the cellulose is high at the lowest WPG. Raising WPG reduces OH stretching in the cellulose. While slight change occurs at the highest WPG. There is no significant increase in the non-acetylated wood.

b- Wave numbers 1380-1375 cm^{-1} represent CH_2 bending vibration in cellulose (Takahashi *et al.*, 1989; Rodrigues *et al.*, 1998).

The CH_2 bending vibration increases in all types of the acetylated wood. However, it is high at the lowest WPG and slight raise occurs at the highest WPG. There is also slight increase in the non-acetylated

wood. The raise in CH_2 vibration is due to the deacetylation process that is located on C_6 of cellulose polymer.

Due to the deacetylation process in the acetylated wood, acetyl groups substitute with OH groups from surrounding wet environment. It indicates well protection of cellulose due to the acetylation.

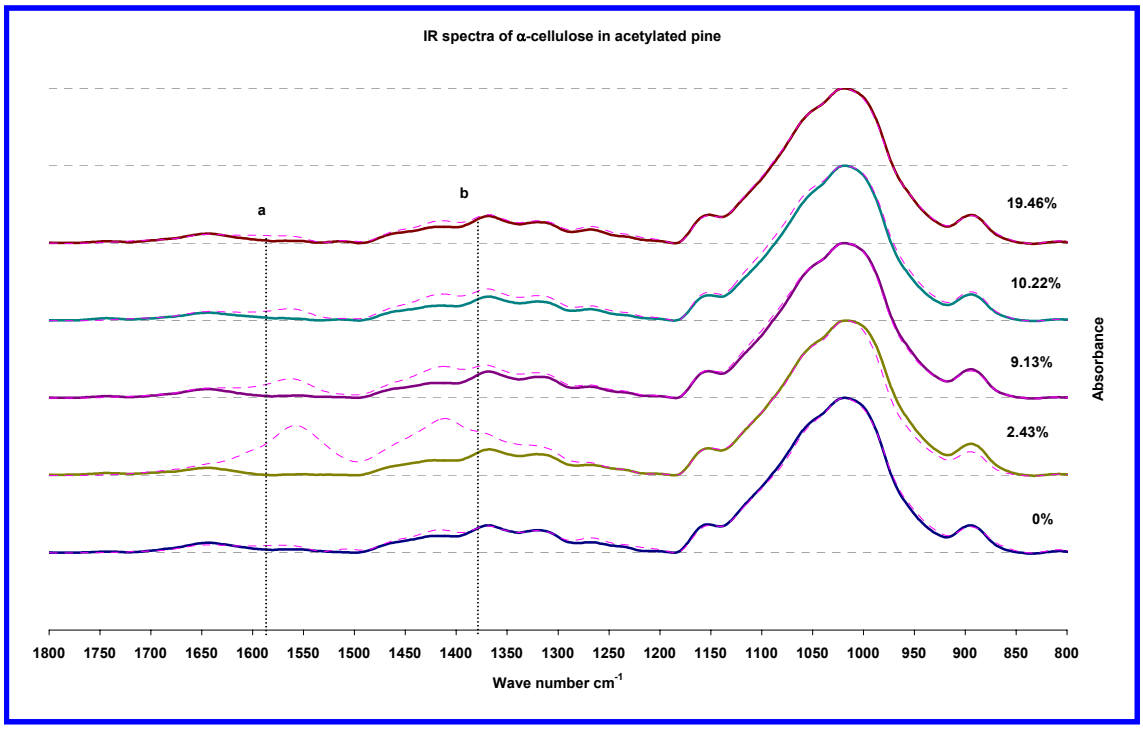

Fig. 2-25- IR spectra of α-cellulose in acetylated Scots pine wood: bold lines: before exposure to soil; doted lines: 300 days after exposure to soil: Wave numbers.

a: 1600-1590cm^{-1}; b: 1380-1375cm^{-1}

2.4.5. Microcalorimetry

Thermal activity and energy production in the acetylated beech and pine wood, which were exposed to soil microorganisms during 300 days, are shown in figures 2-26 & 2-27. In both species, thermal power and produced energy during microbial activities were decreased with raising weight gains due to lesser biological activities in the acetylated wood. Microorganisms are more active biologically in the non-acetylated wood than the acetylated wood and produce more heat and energy than those. At higher weight gains, soil microorganisms were active restrictedly and produce lesser amount of heat. It seems that fungal hyphae were probably dead after colonization during early period of their penetration into the wood due to lack of nutrients in the highly acetylated wood. Heat absorption at the highest weight gain in pine could be related to moisture in the wood or sealing the vials. Probably used vials were not properly sealed and a part of energy was escaped. It has also been reported that adding water to soil reduces heat production (Ljungholm *et al.*, 1979). Wet condition, evaporation and condensation processes cause an abiotical heat change in soil.

Comparison between beech and pine wood revealed that microorganisms produced lower heat in pine than beech. It could be concluded that microorganisms degrade pine wood limitedly due to its higher bioresistance against the microorganisms. It has also been reported that beech is more susceptible to fungal decay than pine wood in the same conditions due to its different chemical and anatomical structure (Butcher & Nilsson, 1982).

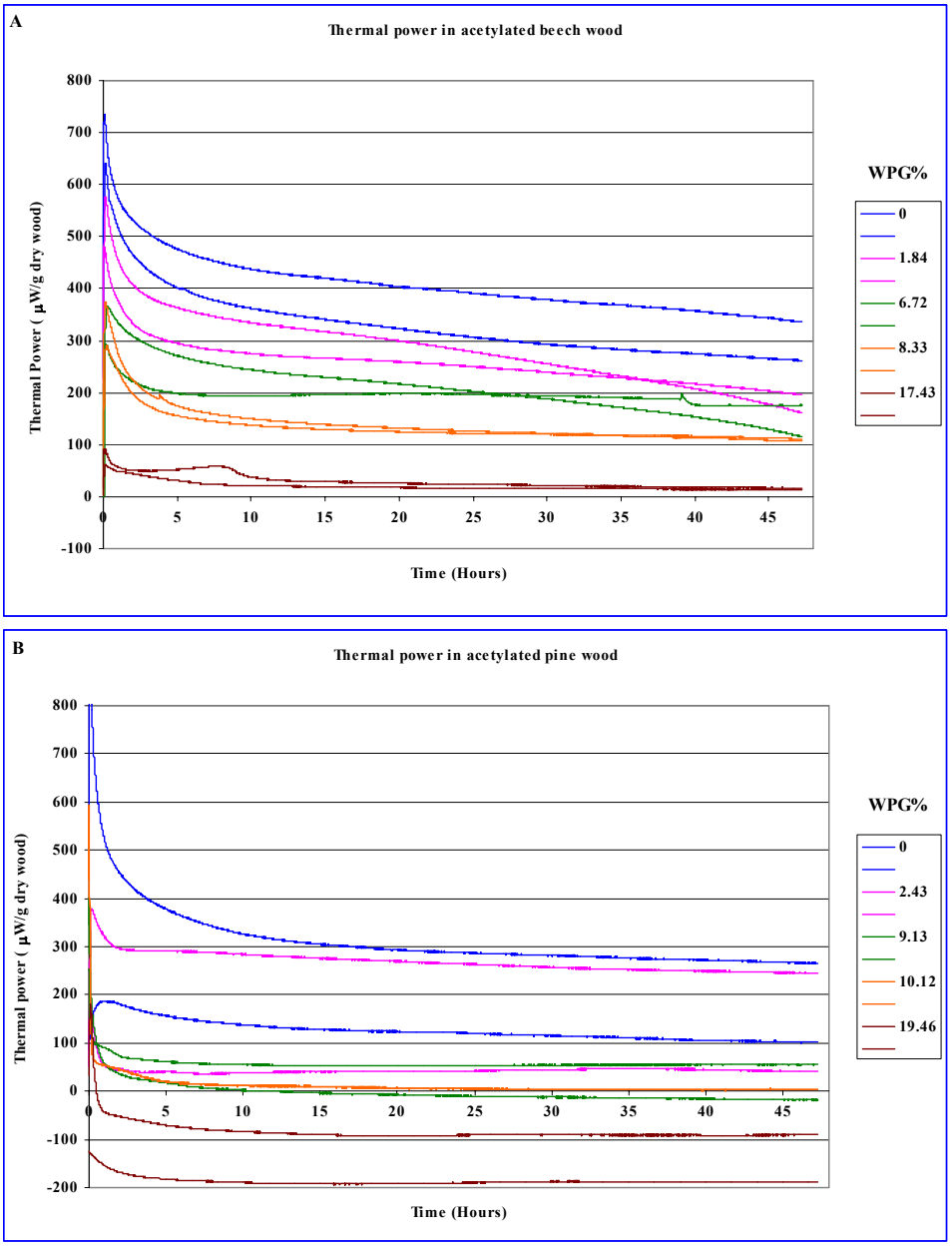

Figure 2-26- Thermal activity in non-acetylated and acetylated beech (A) and Scots pine wood (B)

2.4.6. Ergosterol assay

Figures 2-28 & 2-29 show the amount of ergosterol in the acetylated and the non-acetylated beech and Scots pine wood. The amount of ergosterol in all types of wood raises during the first and second month of exposing to the soil and then it reduces. Also they indicate a secondary raise in its amount, especially in the non-acetylated wood, which is higher in beech wood than pine. Comparison between the acetylated wood with the non-acetylated wood reveals that the amount of ergosterol in the non-acetylated wood is higher than the acetylated one and it reaches approximately nil at the highest weight gains.

First raise in the amount of ergosterol indicates the *colonization* of fungi in wood, while they assimilate organic compounds from the soil and grew rapidly. After using the major amount of nutrients in the soil, the amount of ergosterol decreases due to insufficiency of food, because fungi metabolize their reserved

ergosterol in mycelial walls to survive due to deficit of the nutrients (Braun-Lüllemann, 1989). Then fungi begin to search for required nutrients in wood by producing their enzymes and degrading the wood. Due to use of cell wall polymers in wood, the amount of mycelial biomass increases for second time, especially in the beech wood. It seems that the major degradation of wood occurs during second phase, which is possible to be named *degradation* phase. Anatomical structure of the beech wood such as open vessel lumina and readily available nutrients in ray cells may have contributed to the early and rapid colonization of the fungi through the vessels, ray cells and axial parenchyma in the beech wood and also in pine through the ray cells and resin canals. An early and rapid colonization and then reduction of a blue stain fungus, *Lasiodiplodia theobromae*, has been reported in birch and pine by measurements of ergosterol (Encinas & Daniel, 1999) and the same was reported for *Ceriporiopsis subvermispora* (Messner *et al*, 1998).

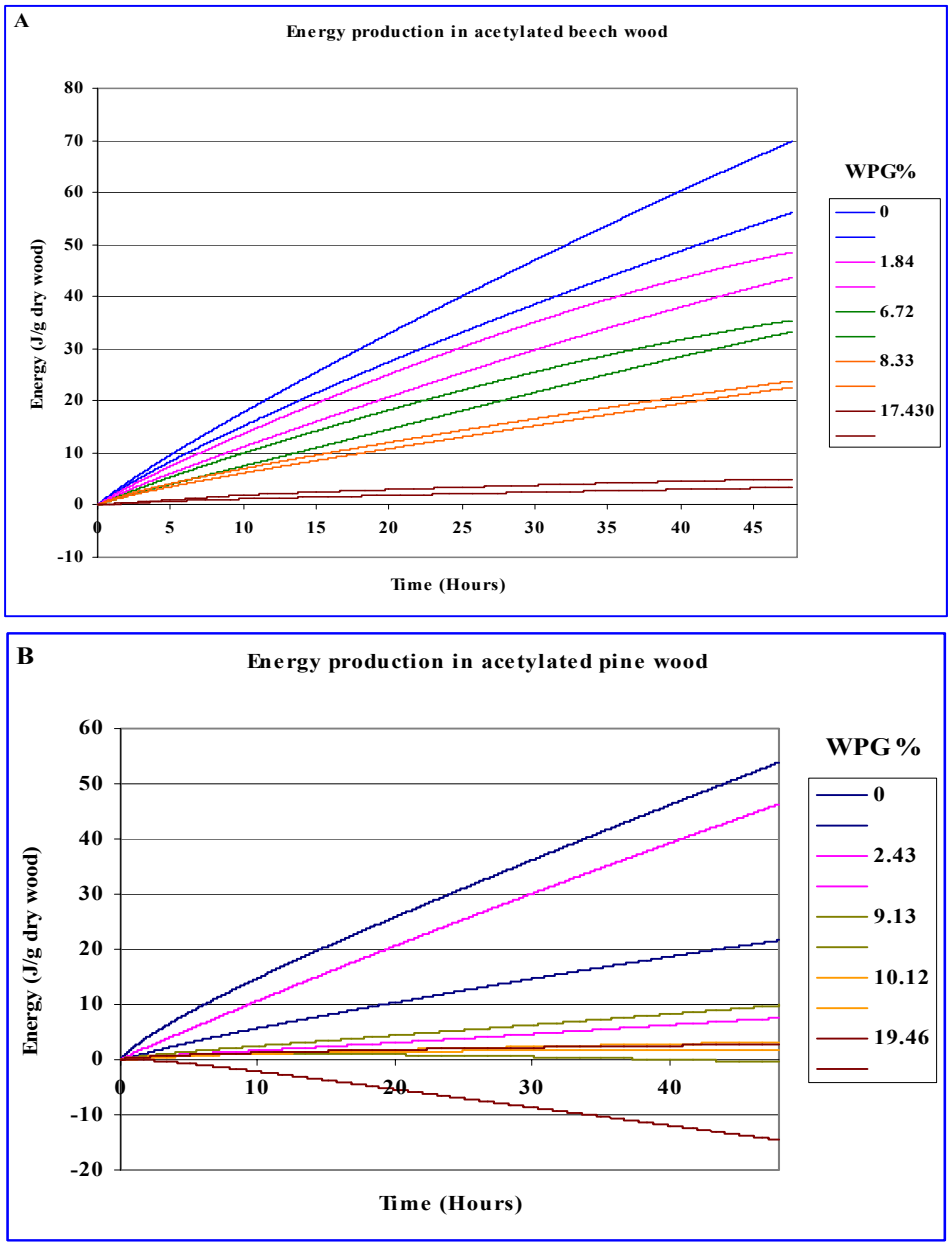

Fig. 2-27. Total produced energy in non-acetylated and acetylated beech (A) and Scots pine wood (B)

Regarding the above explanation, the colonization phase occurs in the non-acetylated and the acetylated wood. However, fungal biomass reduces with raising the weight gains and reaches almost nil at the highest weight gains in both wood. Due to inaccessibility of wood polymers and lack of nutrients in the acetylated wood at higher weight gains, fungi have no possibility to find their necessary nutrients in wood and have to autolyze their reserved ergosterol in the mycelial walls. Finally fungi die due to lack of nutrients and their non-active hyphae remain in the cell lumina. The resulted raises of mass and chemical components in the highly acetylated wood probably are due to the dead mycelial masses that were left after the colonization phase in the wood. The presence of fungal mycelia was proved by above micrographs. It has also been reported that dead mycelia do not produce ergosterol (Braun-Lüllemann, 1989) and ergosterol indicates live fungal mass because of its localization in membranes and oxidation upon cell death (Gessner & Schmitt, 1996).

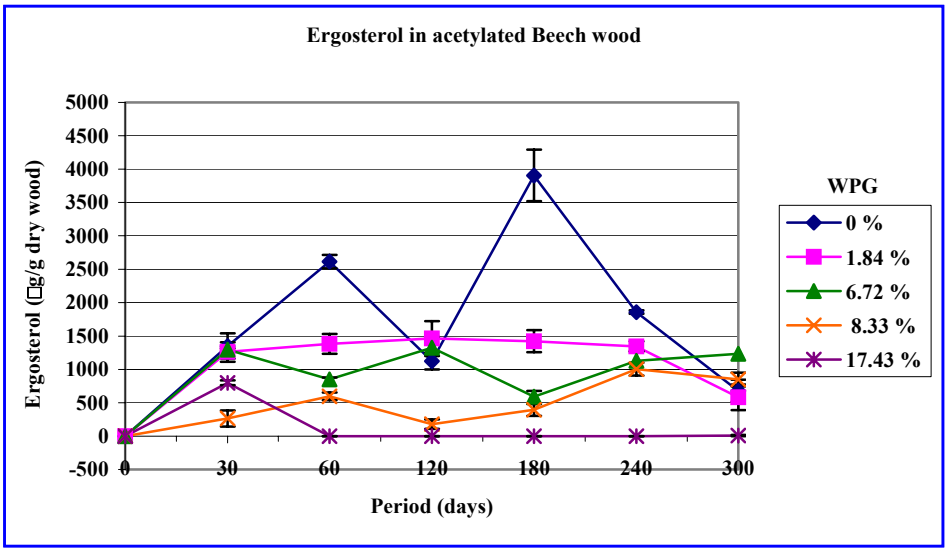

Figure 2-28. Ergosterol content in acetylated beech wood

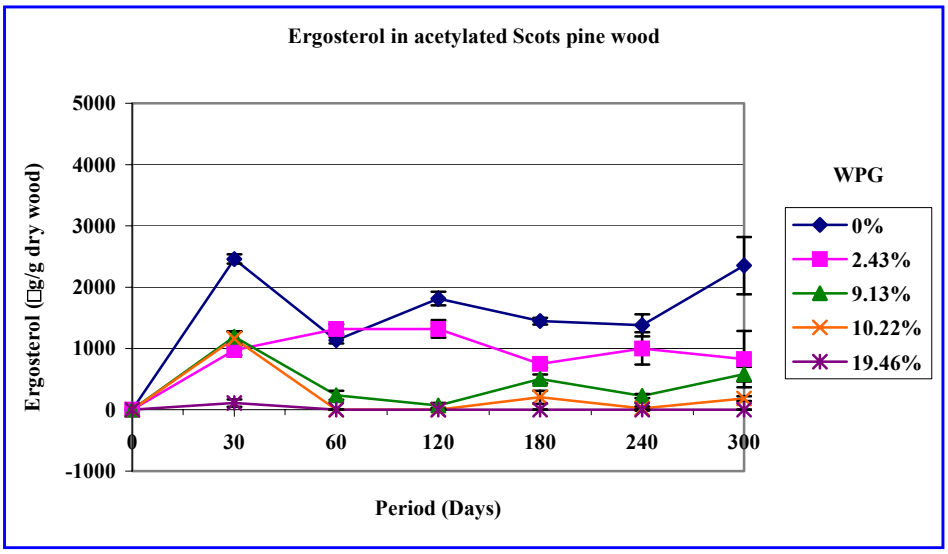

Figure 2-29. Ergosterol content in acetylated Scots pine wood

2.5. General conclusion

Our observations and measurements indicate that the acetylation gives a good protection to the wood against soil microorganisms above 10% of weight gains. Mass and MOE_{dyn} tests showed that soil microorganisms could not decay the acetylated wood at the higher weight gains. Microscopy also revealed that fungi can colonize within the cell lumina and rays without any decay at the higher weight gains and then die due to the lack of nutrients. Microcalorimetry indicated lower heat production due to fungal activities at lower weight gains and no or slight heat production at the higher weight gains. Ergosterol determination also showed no bioactive fungal masses at the higher weight gains in the acetylated wood. Chemical analysis also revealed that soil microorganisms could not remove cell wall components at the higher weight gains. This protection obtains due to substitution of the hydroxyl groups in cell wall polymers by the acetyl groups. It could be concluded that active sites in wood polymers are blocked due to this substitution and are not accessible for soil microorganisms. Raising the degree of acetylation brings more blocked sites and gives higher bioresistance to wood.

2.6. Acknowledgements

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Chapter 3

Microbial attack of acetylated wood in field soil trials

3.1. Introduction

Microorganisms can degrade wood under varied environmental conditions. However, some wood degrading microorganisms, such as soft rot fungi, can tolerate a wide range of temperature, humidity and pH conditions, others have limited tolerance. This also applies to the composition of wood substrates, such as the type and amount of lignin in wood cell walls and the amount and toxicity of extractives present in wood (Eriksson *et al.*, 1990).

Although both fungi and bacteria can degrade wood, fungi, particularly basidiomycetes, are usually more aggressive than bacteria, which are also considered to degrade wood slowly. However, with exception of soft rot fungi, which in nature are often present with bacteria in wood, bacteria are more tolerant to extreme conditions than fungi, such as high lignin and extractive content in wood, high preservative loading, and low levels of oxygen, etc (Rowell & Barbour, 1990; Liese *et al.*, 1995; Kim & Singh, 1993).

Under conditions which may lack oxygen (anaerobe) or where little oxygen (facultative anaerobe) is present, such as in deep mud, on ocean floors and deep waters, the wood is primarily degraded by bacteria (Singh *et al.*, 1991&1995; Kim *et al.*, 1996; Kim & Singh, 2000). In particular erosion bacteria appear to be most tolerant to such conditions. Similar, but less harsh conditions with regard to the presence of oxygen can also support the activities of tunneling bacteria and soft rot fungi. However, wood destroying fungi, basidiomycetes, live in conditions with less moisture and oxygen.

Acetylated wood has been tested under laboratory conditions to evaluate the influence of the acetylation on fungal activities (Rowell *et al*, 1989; Beckers & Militz, 1994; Larsson *et al.*, 1997; Larsson, 1997; Takahashi *et al.*, 1989; Takahashi, 1996; Suttie *et al.*, 1997; Nilsson *et al.*, 1988). However, it is unknown whether the acetylated wood can tolerate real field condition, with plenty of different microorganisms that can attack wood separately or cooperatively, or not. Concerning this purpose, a field trial was set up by research team from SHR (Stichting Hout Research) at October 1993 in Schijndel field, The Netherlands. The tests were done according to EN 252 – "Wood preservatives- Determination of the relative protective effectiveness in ground contact – Field test methods" and prolonged for 350 weeks (7 years) to evaluate the effect of the acetylation against soil microorganisms in a real condition. Our sampling carried out to visualize wood degradation in microscopical level after about 5 years of exposure (at 1999).

3.2. Material and methods

3.2.1. Sample preparation

Test stakes of 25×50×800mm (R×T×L) were prepared from beech (*Fagus sylvatica*), Scots pine (*Pinus sylvestris*) and poplar (*Populus sp.*) and conditioned at 25°C and 65% of relative humidity to moisture content of 12%.

3.2.2. Acetylation

Conditioned wood stakes were acetylated in a stainless steel reactor at SHR (Stichting Hout Research, The Netherlands). The acetylation procedure consisted of 1.5 hours vacuum of 0.04MPa while heating the reactor. The reactor was filled with acetic anhydride and pressurized for one hour. Pressure ranged from 6.5MPa for beech and poplar to 8 MPa for Scots pine. After impregnation the reactor was drained to achieve three different degrees of acetylation (table 3-1).

Un-reacted acetic anhydride and by-product acetic acid were removed from the reactor by evacuating the reactor while still heating. Samples treated at 80°C were rinsed in water for 7 days to convert all untreated anhydride into acid. The other samples were air dried for 3 days. All samples were dried in an oven for 10 days while increasing the temperature from 40 to 100°C. Degree of acetylation was calculated as weight gain (WPG) using the same method as described in chapter 2. In each batch 24 stakes of one wood species were treated. Twenty stakes of each batch were used for the field trial test and 4 samples were

used for acetyl content determination. They were randomly selected to represent an average of the whole batch.

Table 3-1. Applied conditions during the acetylation procedure

Acetylation process	Temperature (°C)	Time (h)
A	80	3
B	120	3
C	120	16

3.2.3. Determination of acetyl content

As Beckers *et al.* (1995)[1] described, a section of 100mm was cut from the middle of the sample and divided into 3 sections of 5mm each for analysis. Acetyl content was determined with HPLC using an Aminex HPX-87H column with a mobile phase 0.005M sulfuric acid. However weight percent gains (WPGs) are used here to indicate degree of acetylation. The average of achieved WPGs is listed in table 3-2.

Table 3-2. Measured WPGs in stakes

Acetylation process	Average WPG (%)		
	Beech	Scots pine	Poplar
Control	-	-	-
A	8.3	8.0	16.8
B	9.6	9.8	-
C	18.8	20.4	18.8

3.2.4. Field test of acetylated wood in soil contact

The test site in Schijndel (5° 26" E, 51° 3" N; The Netherlands) was considered as the test field. The stakes were half buried vertically in soil for 350 weeks (about 7 years, since 1993 till 1999). Sampling from the stakes for microscopy was carried out after 240 weeks of exposure by leaving 80mm of both ends and samples were taken from above ground, ground and below ground levels as shown in fig. 3-1. Small mini-block were cut from the prepared samples and used for microscopy.

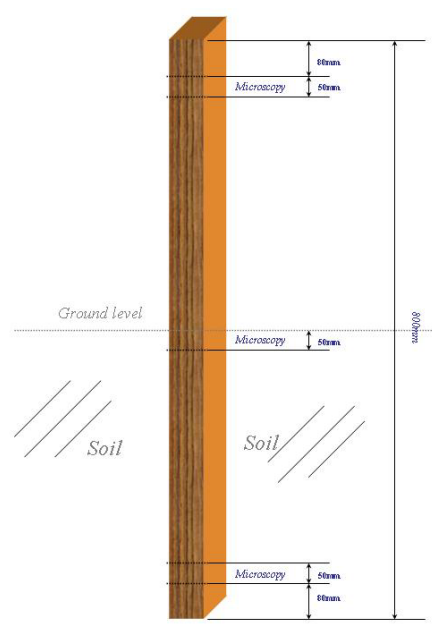

Fig. 3-1. Sampling design through the stakes

[1] This is a part of research that the sample preparations were carried out by Beckers *et al.* (1995). For any details refer to the mentioned article.

3.2.5. Microscopy

3.2.5.1. Light microscopy

Sections were cut from prepared small min-blocks (5×5×10mm) and stained with different stains and mounted with Kaiser's glycerol gelatin. The used stains were Safranin 1% aqueous, Acrindine Orange 0.5% aqueous and Picrin Aniline Blue (Klaassen *et al.*, 2000). The sections were stained for 5-10 minutes. Acrindine orange was used for fluorescent microcopy to indicate any pattern of white rot decay within its early stages. It indicates the decay in brown/red color. Picrin aniline blue visualizes bacteria and fungal hyphae.

3.2.5.2. Scanning Electron Microscopy

The mini-blocks were fixed overnight with 3% glutaraldehyde in 0.1M phosphate cacodylate buffer. Then they were rinsed in the same buffer three times for 30 minutes. Post-fixation was carried out with 1% osmium tetra-oxide in 0.1M phosphate cacodylate buffer for 4 hours. Fixed specimens were washed by distilled water three times for 30 minutes. Dehydration with ethanol series (10, 30, 50, 70, 80, 90 and three times 100%) was carried out for 30 minutes (each step). Critical point drying was applied with CO_2 at 42°C. The specimens were coated with Au/Pd at 0.4 torr pressure and 20mA for 3min. And finally they were examined under a Jeol JSM-5200 SEM at 15-20 kV.

3.3. Results

Beech- Figures 3-2 to 3-5 demonstrate microbial degradation in non-acetylated and acetylated beech wood after five years exposing to field soil. Different types of microbial decays were observed in the non-acetylated and also acetylated beech wood. In the non-acetylated beech wood severe white rot (fig. 3-2 A&B) and soft rot decay (fig. 3-2D) were indicated. Bacterial degradation was also distinguished only in vessel pits (fig. 3-2C). In moderately acetylated beech wood (WPG 8.3%), soft rot decay was distinguished as dominant pattern (fig. 3-3A&B) and also bacterial decay was observed in vessel pits (fig. 3-3C). At WPG 9.6%, it was observed that white rot decay is developing in fibers from lumina and fungi are slowly removing cell wall components (fig. 3-4A). Typical soft rot decay pattern was also indicated in moderately acetylated beech wood (fig. 3-4B). Bacteria were also observed that are locating in vessel pits (fig. 3-4C). At the highest WPG (18.8%), white rot fungal hyphae colonized into cell lumina and distributing via cell pits (fig. 3-5A). However fluorescent microscopy revealed that white rot decay is still at initial stage (fig. 3-5B) and no considerable pattern of white rot has been observed in the cell walls. While soft rot decay is developing better than other types of decay (fig. 3-5C). No bacterial degradation was indicated at the highest WPG and bacteria are limited into vessel pits with no considerable degradation (fig. 3-5D).

Results of microscopical observation are shown in table 3-3. Table 3-4 indicates the results for strength loss (3 point bending strength) at different sampling time points during 7 years measurements. Concerning these results, it reveals that any loss in strength decreases when WPG increases. An increase in MOE has been measured at the highest WPG.

Scots pine- Figures 3-6 to 3-8 show microbial decay in non-acetylated and acetylated pine. Bacterial degradation (tunneling type, fig. 3-6B) and severe soft rot decay (fig. 3-6A) were observed in the non-acetylated pine. In moderately acetylated pine (WPGs 8.0 & 9.8%), white rot decay was indicated at early stage by fluorescent microscopy (fig. 3-7A-D). Figure 3-7A shows hyphae are located in tracheid cell lumina and no decay could be observed under ordinary light. However, using fluorescent microscopy efforts to indicate that cell walls are modifying by white rot enzymes (fig. 3-7B&D). Figure 3-7B shows developing stage of white rot decay. While figure 3-7D indicates an initial stage of white rot decay in cell walls (WPG 9.8%). Figure 3-7C demonstrates that white rot hypha penetrates through the cell wall to access another cell lumen. Due to enzyme effect S_3 layers are swollen and CML is also decaying.

At WPG 20.4% white rot hypha has produced hyphal tunnel through the cell wall to access lignin rich regions (fig. 3-8A). However, fluorescent microscopy shows initial stages of white rot decay in cell wall (fig. 3-8B). Pits are also in developing stage of white rot decay (fig. 3-8C). Pit borders are dissolving by white rot enzymes (fig. 3-8C). Bacterial decay was also observed in pits (fig. 3-8D).

The results of microscopical studies have been summarized in Table 3-3. MOE measurements are also indicated in table 3-4. MOE loss measurements also show that the acetylation protects wood at higher degrees.

Table 3-3. Microbial decay in acetylated wood

Species	WPG	Microbial decay		
		Soft rot	White rot	Bacterial degradation
Beech	Control	Severe	Severe	Vessel pits
	8.3%	Severe	-	Vessel pits
	9.6%	Developing stage	Developing stage	Vessel pits
	18.8%	Developing stage	Initial stage	Vessel pits
Scots pine	Control	Severe	-	Pits
	8.0%	-	Developing stage	Pits
	9.8%	-	Initial stage	Pits
	20.4%	-	Initial stage	Pits
Poplar	Control	Severe	-	Pits
	16.8%	Severe	Developing stage	Pits
	18.8%	-	Developing stage	Tunneling & Pits

Table 3-4. Loss in modulus of elasticity (3 point bending method*) in acetylated wood

Species	WPG	MOE loss (%) after			
		32 weeks	67 weeks	180 weeks	350 weeks
Beech	Control	23.4	38.8	-	95.2
	8.30%	13.2	22.8	33.2	77.6
	9.60%	10.4	13.7	29.9	83.2
	18.80%	7.8	6.5	7.6	-4.6
Scots pine	Control	16.1	20	35.1	84.9
	8.0%	9.3	13.7	12.6	26.8
	9.80%	9.9	14.9	8.9	14.7
	20.40%	8	8.4	5.7	0.8
Poplar	Control	20.9	43.6	70.3	100
	16.80%	9.1	19.9	21.6	53.9
	18.80%	5.9	7.4	7.6	6.9

- Results are based on the report from SHR (Stichting Hout Research, The Netherlands).

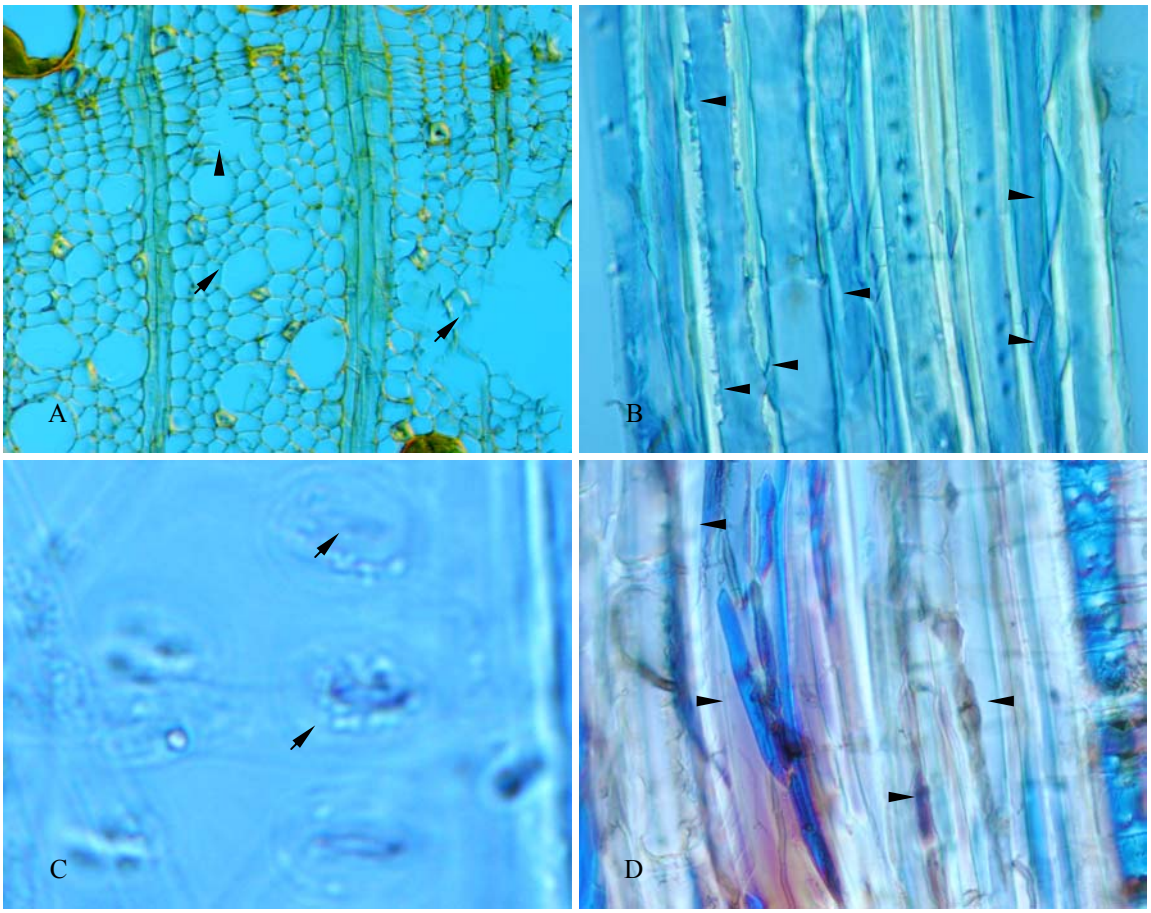

Fig. 3-2. Severe biodegradation in non-acetylated beech. **A&B:** White rot decay (arrow heads); Nomarsky. **A:** Cross section; Nomarsky. **B:** Radial section; Nomarsky. **C:** Bacterial degradation in pits (arrow heads); Radial section, Nomarsky. **D:** Soft rot decay (arrow heads); Radial section, Nomarsky.

Poplar- Biological degradation in non-acetylated and acetylated poplar wood has shown in figures 3-9 to 3-11. Severe soft rot decay was observed in non-acetylated poplar (fig. 3-9A). Figure 3-9B also shows white rot erosions on fiber cell walls. Bacterial degradation was also distinguished in vessel pits of poplar (fig. 3-9C). At WPG 16.8%, soft rot decay was observed in acetylated poplar (fig. 3-10A). Figure 3-10B&C indicates white rot decay pattern in moderately acetylated wood. Also it was observed that white rot fungi are decaying compound middle lamella (fig. 3-10D). Figure 3-11 shows white rot and bacterial decay in highly acetylated poplar wood (WPG 18.8%). Concerning the figure 3-11A&B, it seems that white rot fungi have modified secondary wall and the cell walls were eroded due to enzymatic activities (arrow heads). In figure 3-11A&B, the detachment between middle lamella and secondary wall is seen. They are related to bulking effect of the acetylation process at higher weight gains. Because high numbers of substitutions of hydroxyl groups by acetyl groups occurs during strong acetylation to obtain the highest weight gains. Sequentially the bulking effect on cell wall appears with detachment between ML and secondary cell wall. Rod shape bacteria (flagellate and non-flagellate) were observed in highly acetylated poplar (fig. 3-11C-E). Those bacteria probably belong to tunneling type and stay in cell wall. Figure 3-11E shows a rod shape bacterium has produced vesicles containing enzymes. Bacteria produce enzyme to degrade cell walls.

3.4. Discussion and conclusion

Concerning the observations, soft rot is dominant rot-fungi in field soil and decays non-acetylated wood severely and attack also moderately acetylated wood slowly. Bacteria are also in close association with soft rot fungi to degrade acetylated and non-acetylated wood. In moderately acetylated wood, white rot decay is

at developing stage, while it is at initial stage at the highest WPGs. Performed measurements for strength loss indicates no MOE loss in beech and negligible loss in Scots pine and poplar at the highest WPGs after 350 weeks (7 years). However microscopy reveals that initial stage of decay has began at the highest WPGs and it is at developing stage of decay in moderately acetylated wood. The less development of decay in the acetylated wood comparing the non-acetylated wood proves a very good protection of wood against soil microorganisms due to the acetylation after 7 years period.

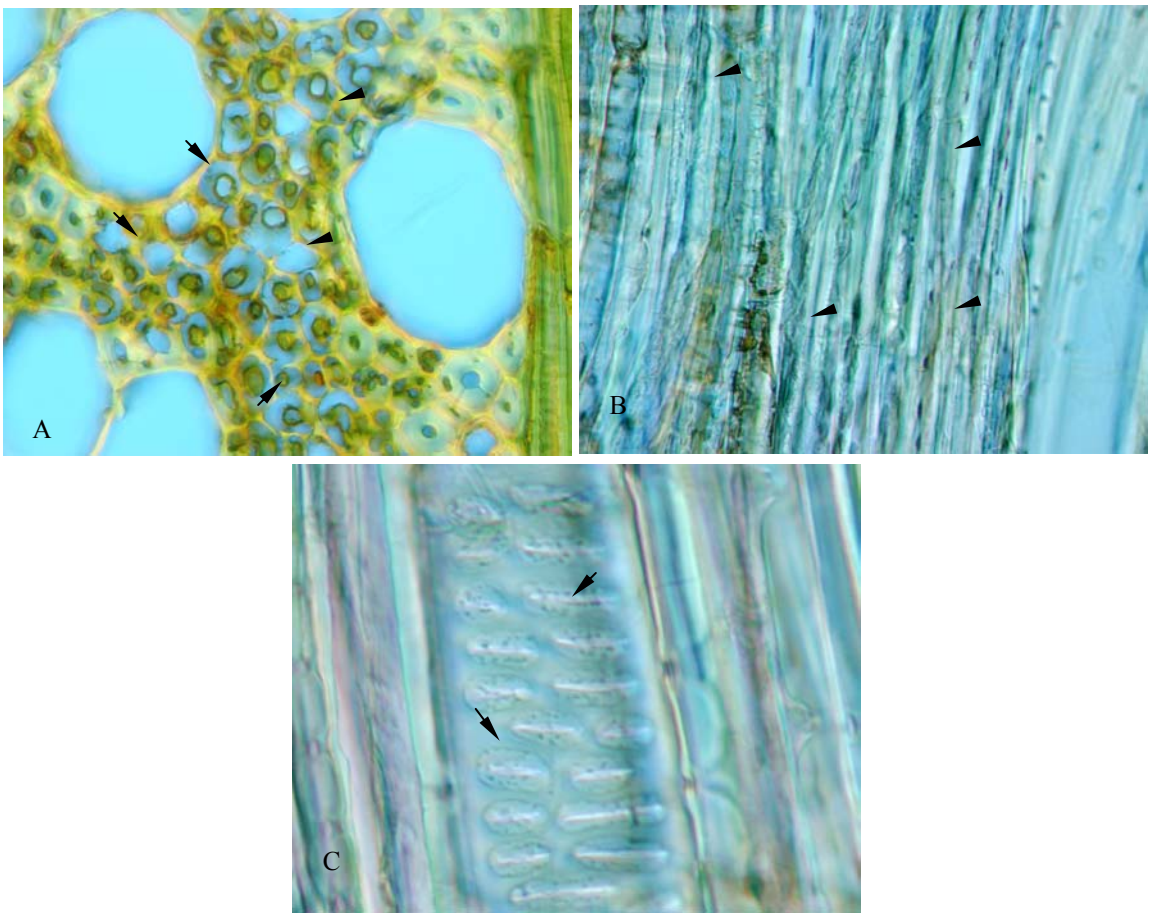

Fig. 3-3. Severe soft rot decay (A&B, arrow heads) and bacterial degradation in vessel pits (C, arrow heads); Nomarsky, WPG 8.3%. **A:** Cross section, Nomarsky. **B&C:** Radial section; Nomarsky.

Soft rot and white rot decays were observed in non-acetylated and acetylated wood at the same time. While no brown rot decay was observed in those woods. Bacteria always were seen in all types of wood. They cooperate generally with soft rot fungi to degrade non-acetylated wood or limit themselves to pits at the higher degrees of the acetylation.

The presence of soft and white rot decays in same samples probably relates to soil moisture, which accelerates due to climatological variation during a year. Because of variable rainfall during a year, low or no rain in summer and high rain in other seasons in The Netherlands, soil gets saturated during all seasons except summer and its moisture reduces during summer. Changing soil moisture affects the oxygen level into the soil. In saturated soil, there is a great limitation of oxygen and restricts aerobic microorganisms and permits anaerobes or facultitative microorganisms. In other words, variation of moisture in the soil affects the moisture content in planted wood samples in the field soil and changing the moisture in the wood samples consequently influences the oxygen level in wood stakes. Oxygen is a very crucial factor that can affect strongly activities of microorganisms in wood (Zabel & Morrell, 1992).

Soft rot fungi activate in a wide range of moisture contents, from relatively dry wood (Daniel & Nilsson, 1998) to saturated condition (Singh & Kim, 1997; Machek *et al.*, 1997; Zabel & Morrell, 1992), whereas optimal wood moisture level for most decay fungi (basidiomycetes) lie between 40 and 80%. And white rot fungi require more moisture than brown rot fungi to achieve optimal wood weight loss (Zabel & Morrell, 1992). Although a moisture content around fiber saturation point (28-30%) was reported for brown rot fungi (Ritsckoff, 1996). Soft rot fungi can also decay wood in a wide range of temperature from 0°C to around 60°C (Daniel and Nilsson, 1998), while the optimum temperature for basidiomycetes is between 24-32°C (Zabel & Morrell, 1992).

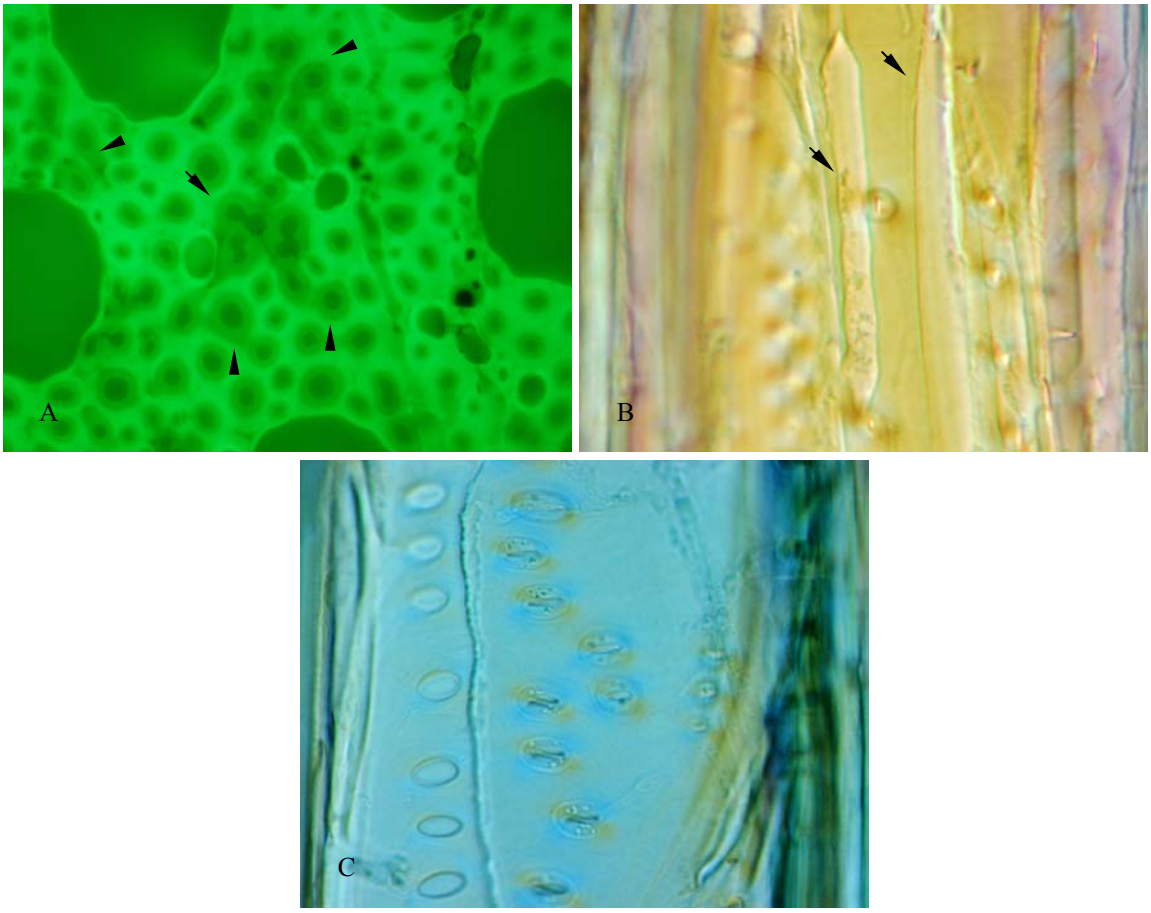

Fig. 3-4. Microbial degradation in acetylated beech wood; WPG 9.6%. **A:** Darker area indicate developing white rot decay (arrow heads); Cross section, Fluorescent light. **B:** Soft rot decay (arrow heads); Radial section, Nomarsky. **C:** Bacteria in vessel pits; Radial section, Nomarsky.

Kazemi *et al* (1998 & 1999) reported that in soft rot fungus *Chaetomium globosum* increasing the moisture content in wood blocks generally cause weight losses on beech and Scots pine wood to be increased. Reduced oxygen level appears to cause much smaller reduction in decay by *C. globosum* than with the basidiomycetes (*Coniophora puteana* and *Coriolus versicolor*). Oxygen plays a clear role on brown rot decay process.

Concerning the above reports, soft rot fungi has good capability to adopt to accelerated soil moisture and temperature to decay wood during whole period of a year, while white rot fungi can decay wood during a short period when the temperature and moisture is in optimal level, e.g. summer, end of spring and early autumn seasons. It seems soil moisture in Schijndel field is higher than the optima for brown rot fungi to activate in wood. Therefore no brown rot decay has been distinguished in wood stakes due to high moisture content and low level of oxygen. Considering the table 3-3 rot fungi were very active in hard wood species,

beech and poplar, while less decay has distinguished in Scots pine wood. According to discussion in chapter 2 about soil bed trial, lignin type is a very important reason to produce possibility of preferential degradation. Levi & Preston (1965) reported that soft rot fungi decay hardwoods more preferable than softwoods. Because hardwood lignin is more methoxylated than softwood lignin (Levi & Preston, 1965).

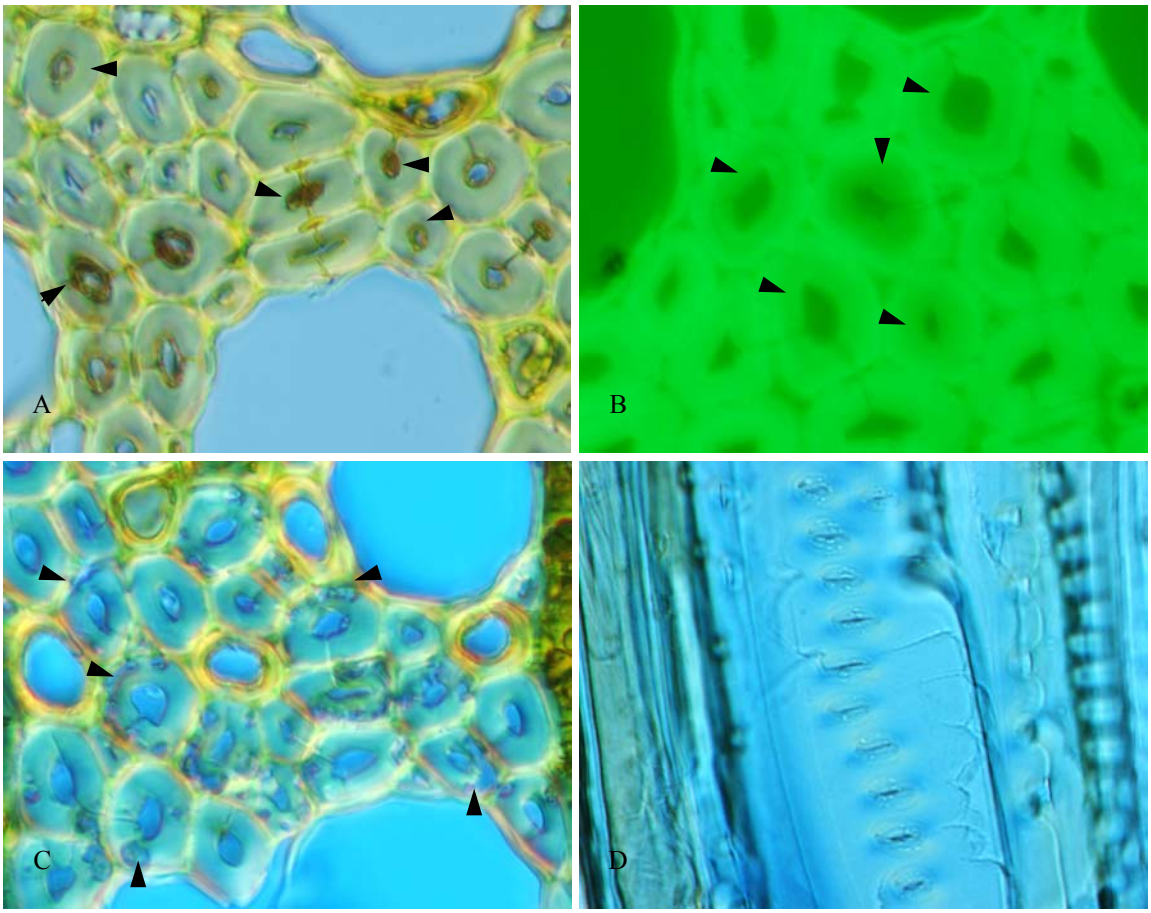

Fig. 3-5. Microbial decay in highly acetylated beech wood; WPG 18.8%. **A&B:** White rot decay at early stage. **A:** White rot hyphae colonize in cell lumina and distribute through cell pits (arrow heads). Still no decay is seen under ordinary light; Cross section, Nomarsky. **B:** Fiber cell walls are modifying and decaying (arrow heads) due to white rot enzymes effect; Cross section, Fluorescent light. **C:** Typical soft rot cavities (arrow heads); Cross section, Nomarsky. **D:** Bacteria present in vessel pits; Radial section, Nomarsky.

On the other hands, soil pH is another reason, which can influence fungal growth. Reports show that brown rot fungi prefer lower pH and white rot fungi grew at higher than the optimal pH for those fungi. Soft rot fungi are also active at pH close to neutral (Zabel & Morrel, 1992). Probably in the test field, soil had not optimal pH for brown rot fungi and there was optimal condition for soft- and white rot fungal growth. Therefore, it could be concluded that soil type, moisture variation, oxygen level and wood species were the main reasons that influenced microbial growth in acetylated wood samples from the test field.

Successional changes in colonizing of wood could probably be another reason for different microbial degradation that was occurred in the wood stakes. Käärik (1975) reported successional changes in the colonizing microorganisms from the initial phase of bacteria, moulds, blue stain and soft rot fungi to the take-over by basidiomyceteous fungi and also successive changes in the attacking decay fungi.

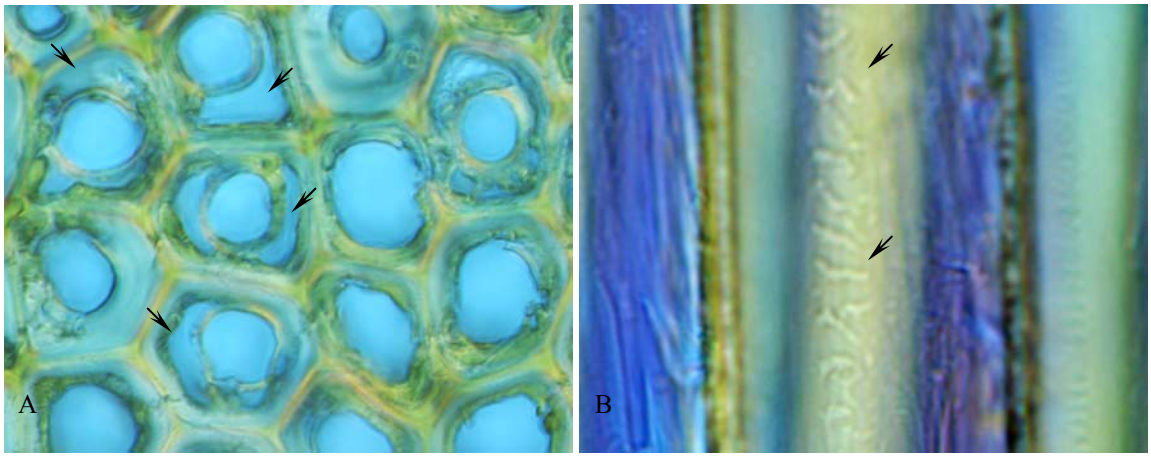

Fig. 3-6. Severe soft rot decay (A) and tunneling bacterial degradation (B) in non-acetylated Scots pine wood. **A:** Secondary wall has extensively removed (arrow heads); Cross section, Nomarsky. **B:** Bacterial troughs (arrow heads); Longitudinal section, Nomarsky.

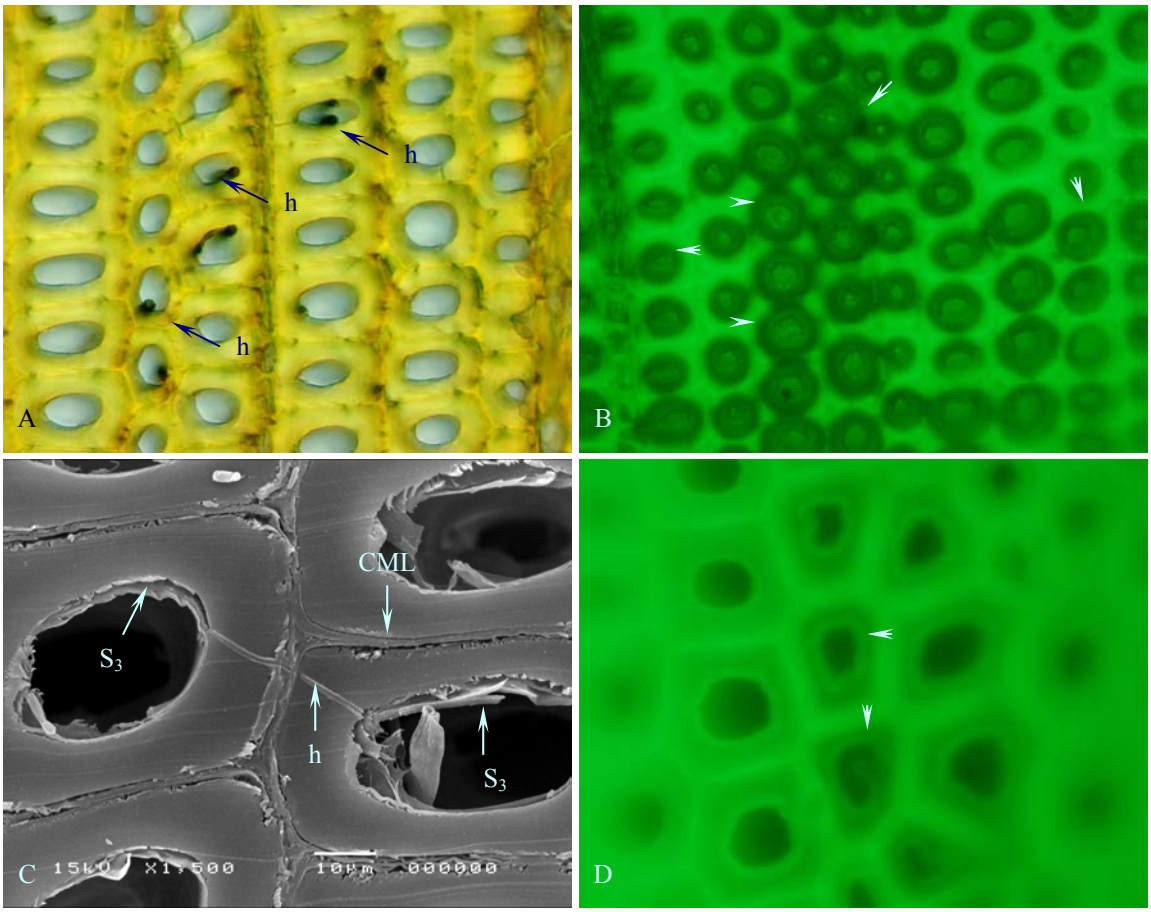

Fig. 3-7. White rot decay in moderately acetylated pine wood. **A-C:** WPG 8.0%; **D:** WPG 9.8%. **A:** Hyphae (h) exist in cell lumina; while no decay pattern is seen in cell walls in normal microscopy; Cross section, Nomarsky. **B:** White rot decay at developing stage (arrow heads); cross section, Fluorescent light. **C:** SEM micrograph shows a hypha (h) penetrates through the cell wall. S_3 layer has been swollen due to enzymatic effects and CML is decaying; Cross view, SEM. **D:** White rot decay at initial stage (arrow heads); Cross section, Fluorescent light.

Our results also showed that at the highest WPGs fungal hyphae can colonize into cell lumina and do not decay wood considerably. These observations also confirm findings of Kumar (1994) that decay hyphae limit themselves to parenchyma cells and tracheid lumina and fail to attack the acetylated cell walls.

According to our observations and MOE loss measurements, we can here conclude that acetylation provides an excellent bioresistance in wood against soil microorganisms and retards their activities for a long period at higher WPGs.

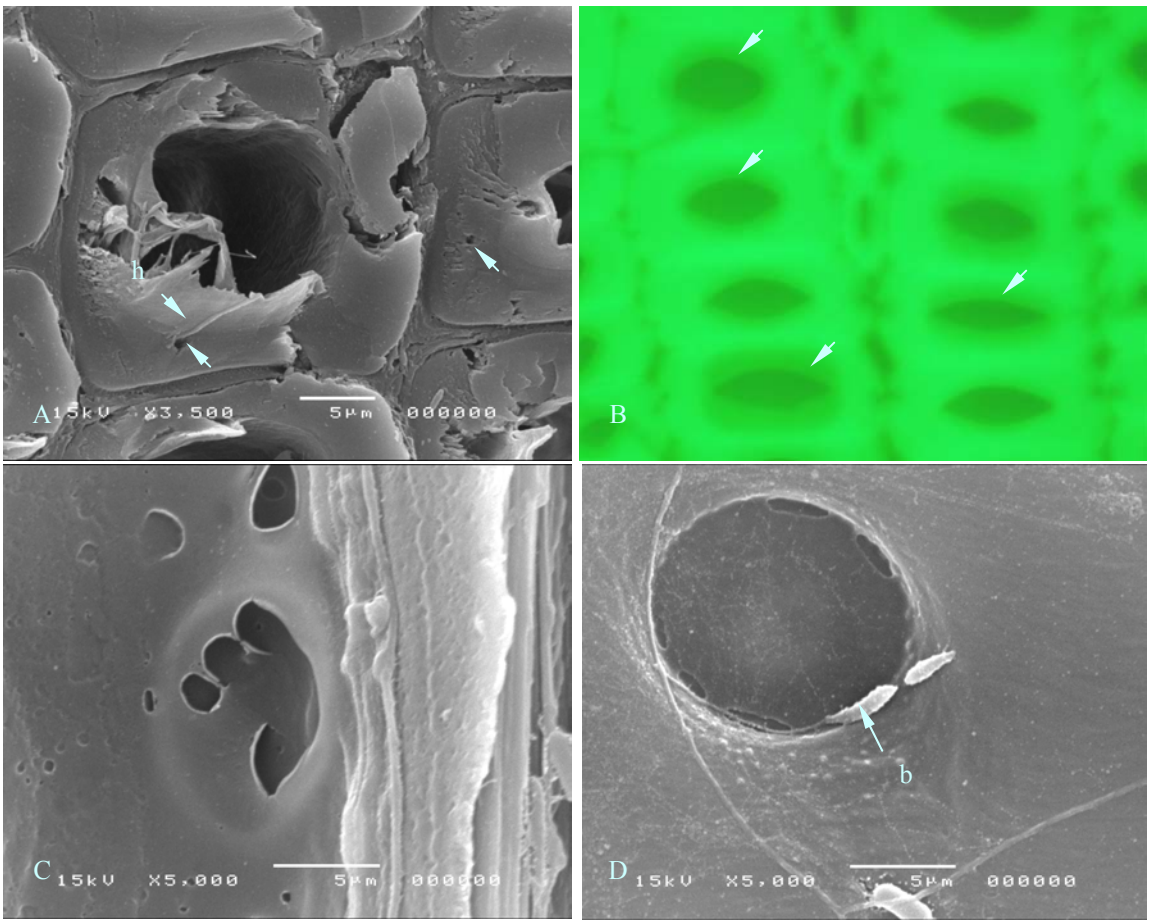

Fig. 3-8. Microbial degradation in highly acetylated pine; WPG 20.8%. **A-C:** White rot decay. **A:** Hypha (h) produces tunnel (arrow heads) into the cell wall; Cross view, SEM. **B:** Initial stage of white rot decay (arrow heads); Cross section, Fluorescent light. **C:** White rot erosion in pit; Radial view, SEM. **D:** Flagellate bacteria (b) decay pit; Longitudinal view, SEM.

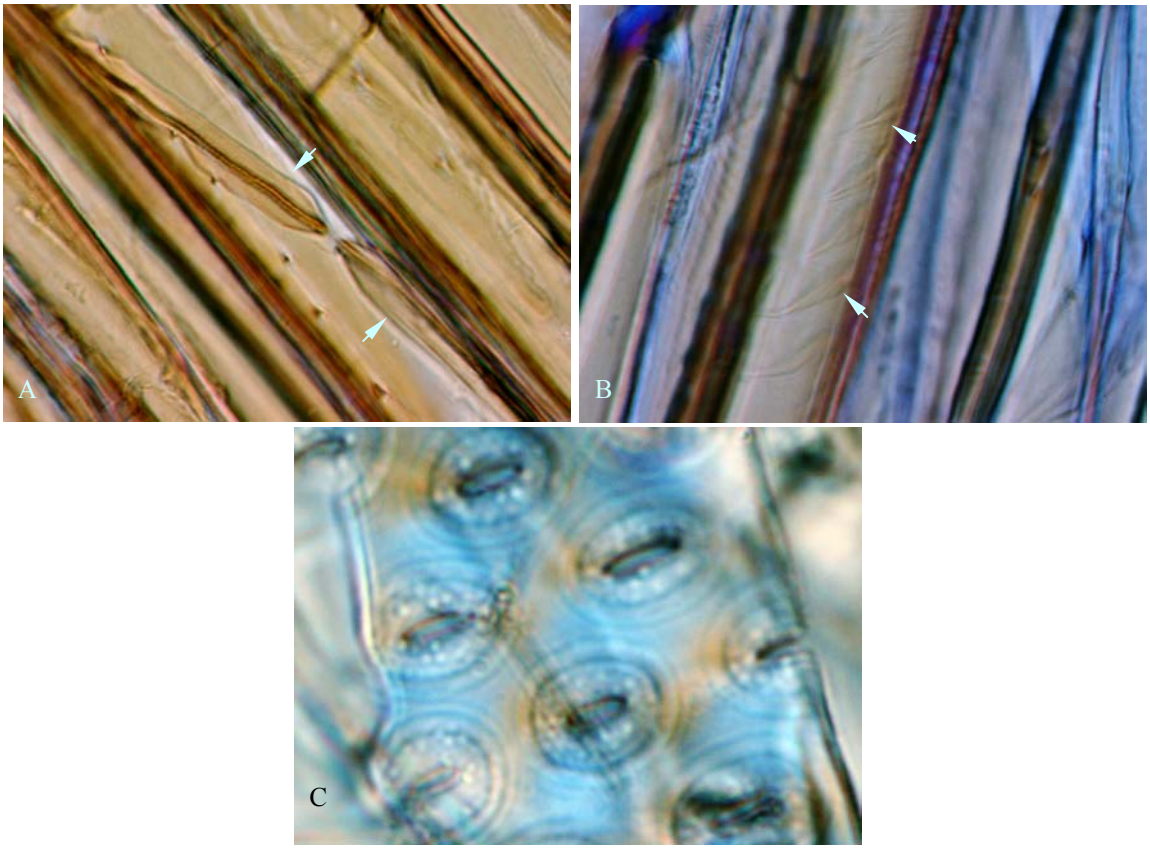

Fig. 3-9. Microbial decay in non-acetylated poplar. **A:** Soft rot decay (arrow heads); Radial section, Nomarsky. **B:** White rot erosion (arrow heads); Radial section, Nomarsky. **C:** Bacterial degradation in vessel pits; Radial section, Nomarsky.

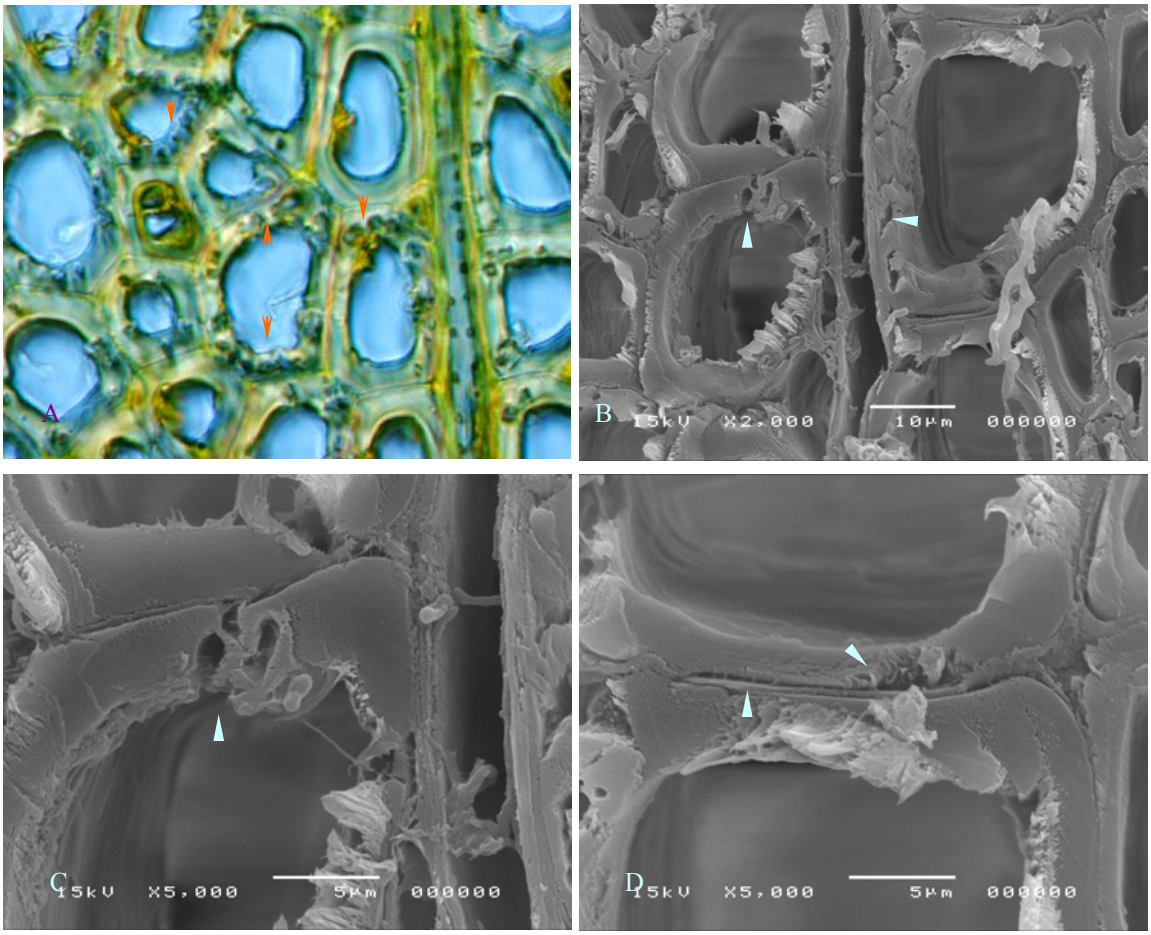

Fig. 3-10. Soft and white rot decay in acetylated poplar; WPG 16.8%. **A:** Typical soft rot decay in fiber cell walls; Cross section, Nomarsky. **B&C:** White rot erosion on fiber cell wall (arrow heads). **D:** Decaying CML (arrow heads). **B-D:** Cross view, SEM.

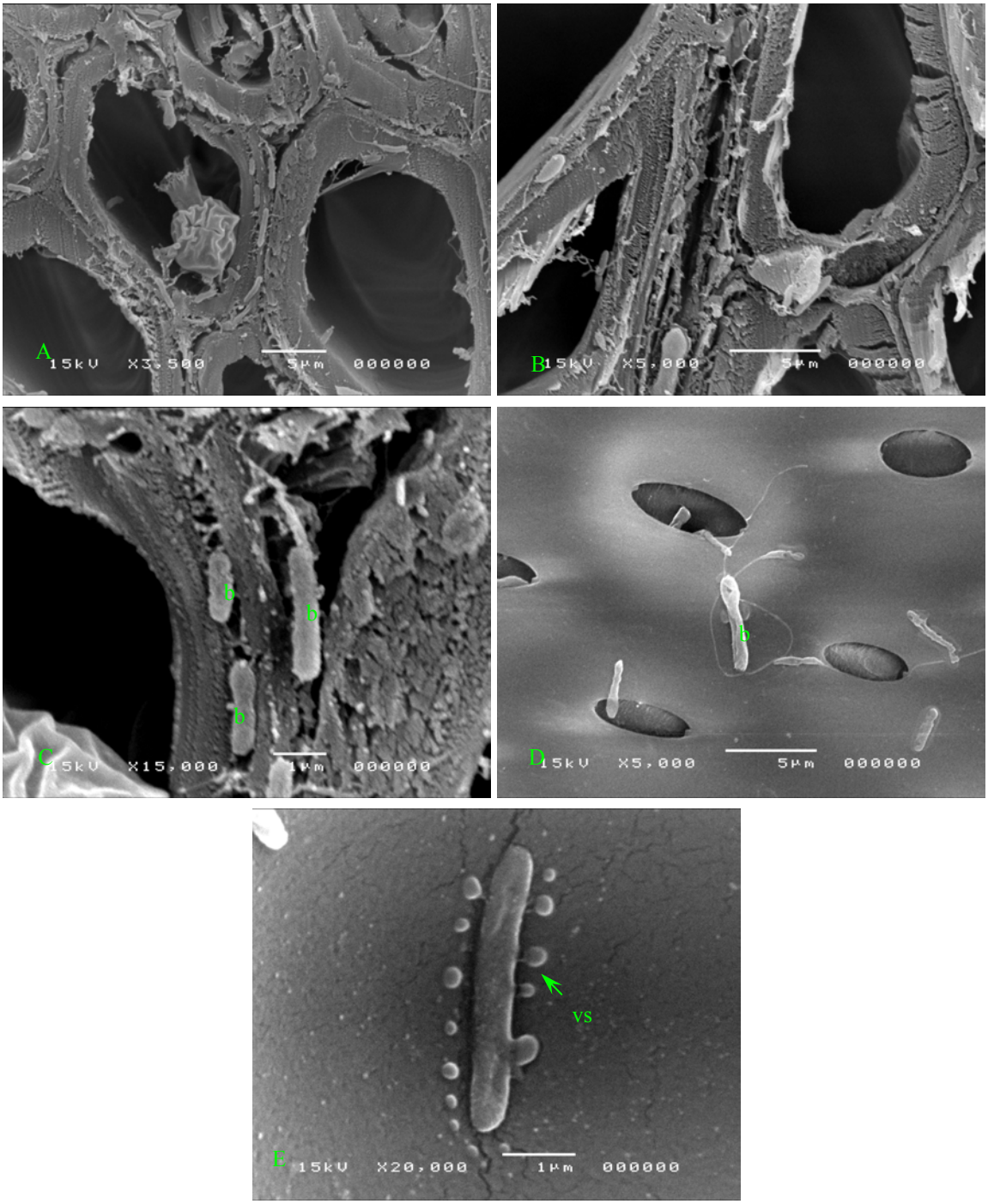

Fig. 3-11. Microbial decay in acetylated poplar; WPG 18.8%. **A-C:** White rot erosion in fiber cell wall. Bacteria (b) are seen in degrading fiber walls; Cross view, SEM. **D:** Flagellate bacteria (b) on vessel pits; Radial view; SEM. **E:** Bacteria produce vesicles (vs) containing enzymes to erode cell wall; Longitudinal view; SEM.

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Chapter 4

Degradation of acetylated wood by Basidiomycetes

4.1. Introduction

The influence of the acetylation on wood properties has been studied and numerous reports have been published in this field. Many of them report that the acetylation provides a good bioresistance in wood and extends its durability with increasing the degree of the acetylation. Majority of the researches are focused on changes in dimensional properties (Goldstein *et al.*, 1961; Youngquist *et al.*, 1986; Rowell *et al.*, 1993), weight loss (Beckers & Militz, 1994; Larsson *et al.*, 1997), strength loss (Ramsden *et al.*, 1997; Dhamodaran, 1995; Rowell *et al.*, 1988), effects on musical tune (Yano *et al.*, 1993; Yano & Minato, 1993), etc. One of the most important focused researches and interests in this field is about bioresistance of wood. Many published reports indicate the effective influence of the acetylation in increasing wood durability (Peterson & Thomas, 1978; Beckers *et al.*, 1995; Larsson *et al.*, 1997; Suttie *et al.*, 1997&1998). Concerning those reports, there is a lack of knowledge on the mode of action on detailed microscopical level to evaluate morphology of decay, stage of decay and patterns of decay in the acetylated wood.

This part of research has been conducted to understand the protection mechanism of the acetylation and to visualize the resistance of the acetylated wood in detailed microscopical level and answer to the questions if there is any difference between the mode of fungal action in acetylated and non-acetylated wood and to find the stages of decay advancement in the acetylated wood. Standard white- and brown-rot fungi (according to European Standard EN 113) have been used to find answers for those questions.

4.2. Material and methods

4.2.1. Sample preparation

Mini-blocks of beech (*Fagus sylvatica*) heartwood and Scots pine (*Pinus sylvestris*) sapwood with sizes 5mm × 5mm × 20mm were prepared and acetylated under the same conditions that were explained in section 2-2. The obtained weight percent gains are listed in table 4-1. Specimens were dried to reach same moisture content before experiment. Thereafter, they were sterilized at 121°C for 20 minutes.

Table 4-1. Average of WPGs in Beech and Scots pine wood

Acetylation process	Weight percent gain	
	Beech	Scots pine
	%	%
A	0	0
B	3.41	2.71
C	8.84	9.97
D	9.99	10.97
E	17.15	19.65

4.2.2. Microorganisms and conditions

***Microorganisms*:** Strains of typical white rot (*Trametes versicolor*) and brown rot (*Poria placenta*) fungi, which were maintaining at 4°C on peptone yeast extract slants (per liter: 20.0g glucose, 5.0g peptone, 2.0g yeast extract, 1.0g KH_2PO_4, 0.5g $MgSO_4 \cdot 5H_2O$, 15.0g agar) in Wood Science Laboratory (Sub-department of Forestry, Wageningen University, The Netherlands), used as test fungal strains.

Medium: Glucose malt-extract (ME) plates (per liter: 15.0g agar, 10.0g glucose, 4.5g malt-extract) were prepared and autoclaved at 121°C for 20 minutes. The ME plates were incubated at 27°C and 70% relative humidity. Agar plugs (3mm diameter) were punched from the leading edge of the mycelium grown on ME plates and used as inoculums for the experiments (Son *et al.*, unpublished; Dorado *et al.*, 2000).

Sterilized test mini-blocks were introduced to the plates carefully and incubated at 27°C and 70% relative humidity for 80 days. Sampling was carried out during 12 weekly intervals for microscopical studies.

4.2.3. Microscopy

Fungal decay patterns in different WPGs were studied under microscopes. For this purpose, the specimens of each WPG in every sampling period were considered for microscopy.

4.2.3.1. Light microscopy

Cross, tangential and radial sections (10-15μm) were prepared from fresh specimens and stained with a mixture of safranin and astra blue immediately for 10 minutes (Klassen *et al*, 2000; Srebotnik & Messner, 1994) and mounted with Kaiser's glycerol gelatin. Normal light, Nomarsky (Differential Interference Condenser, DIC) and polarized lights were used to study the patterns of decay in acetylated wood.

4.2.3.2. Scanning Electron Microscopy

The mini-blocks were fixed with 3% glutaraldehyde in 0.1M phosphate cacodylate buffer for overnight. Then they were rinsed in the same buffer three times for 30 minutes. Post-fixation was carried out with 1% osmium tetra-oxide in 0.1M phosphate cacodylate buffer for 4 hours. Fixed specimens were washed by distilled water three times for 30 minutes. Dehydration with ethanol series (10, 30, 50, 70, 80, 90 and three times 100%) was carried out for 30 minutes (each step). Then critical point drying was applied with CO_2 at 42°C. The specimens were coated with Au/Pd at 0.4 torr pressure and 20mA for 3min. And finally they were examined under a Jeol JSM-5200 SEM at 15-20 kV.

4.3. Results

4.3.1. *Trametes versicolor*

Beech- Figure 4-1 represents a severe attack in non-acetylated beech wood. Due to extensive simultaneous white rot decay, cell walls were eroded and middle lamellae (ML) were dissolved. The integrity of cells was lost (fig. 4-1 A&B). White rot fungus eroded pits and opened them to facilitate hyphal penetration (fig. 4-1 C&D).

Figure 4-2 also indicates extensive white rot decay in acetylated beech wood at the lowest WPG (3.41%). Typical patterns of advanced stage of white rot decay were seen in this wood. Cells were eroded by severe enzymatic activities. Hyphae eroded cell walls from luminal side inward ML and thinned (fig. 4-2 A). Attacked rays (fig. 4-2 B) and opened pits (fig. 4-2 C) also indicate severe white rot decay at low degree of the acetylation.

In moderately acetylated beech wood (WPG 8.84%), developing decay patterns were distinguished (fig. 4-3). Cell walls were slightly eroded and ML in cells was removed scarcely (fig 4-3 A&B). However white rot fungus attacked them simultaneously by thinning the cell walls and dissolving ML. Rays were also attacked extensively (fig. 4-3 C). AT WPG 9.99%, slight decay was also observed in moderately acetylated beech wood (fig. 4-4). Hyphae were colonized into vessels and eroded fiber cell walls simultaneously after penetration into fiber lumina (fig. 4-4 A&B). Not only white rot hyphae erode cell walls when they are located in lumina (fig. 4-4 B), but also they could produce hyphal tunnels to reach to other cell lumina or middle lamellae through the cell walls (fig. 4-4 C).

At the highest WPG of the acetylation (17.15%), no decay patterns were distinguished, while hyphae were colonized in cell lumina and penetrated through pits (fig. 4-5).

Figure 4-6 represents phenology[1] of white rot decay within the incubation period. Concerning this figure, fungal hyphae penetrate into fiber cell lumina and pit chambers in non-acetylated wood and the lowest WPG (3.41%) within first week of the incubation (fig. 4-6A), while at WPGs 8.84%, 9.99% and 17.15% they could penetrate into fiber lumina at second and third week (fig. 4-6A). White rot erosion in cell walls begin at day 19 in the non-acetylated wood and WPG 3.41%. Opening of pits begin at the same time in those woods. Loss of birefringence, a known pattern for decay, occurs after 23 days of incubation. After 23 days, cell walls erosion begins in moderately acetylated wood (WPGs 8.84% and 9.99%) and

[1] Phenology is the study of the timing of occurring and recurring biological phases.

opening of pits also occurs after 23 days at WPGs 8.84% and no openings at 9.99%. While no cell wall erosion and opening of pits were distinguished within the incubation period at the highest WPG (17.15%). Loss of birefringence was observed since the first day of incubation (i.e. sound wood) in both moderately acetylated wood (WPGs 8.84% and 9.99%) and at the highest WPG.

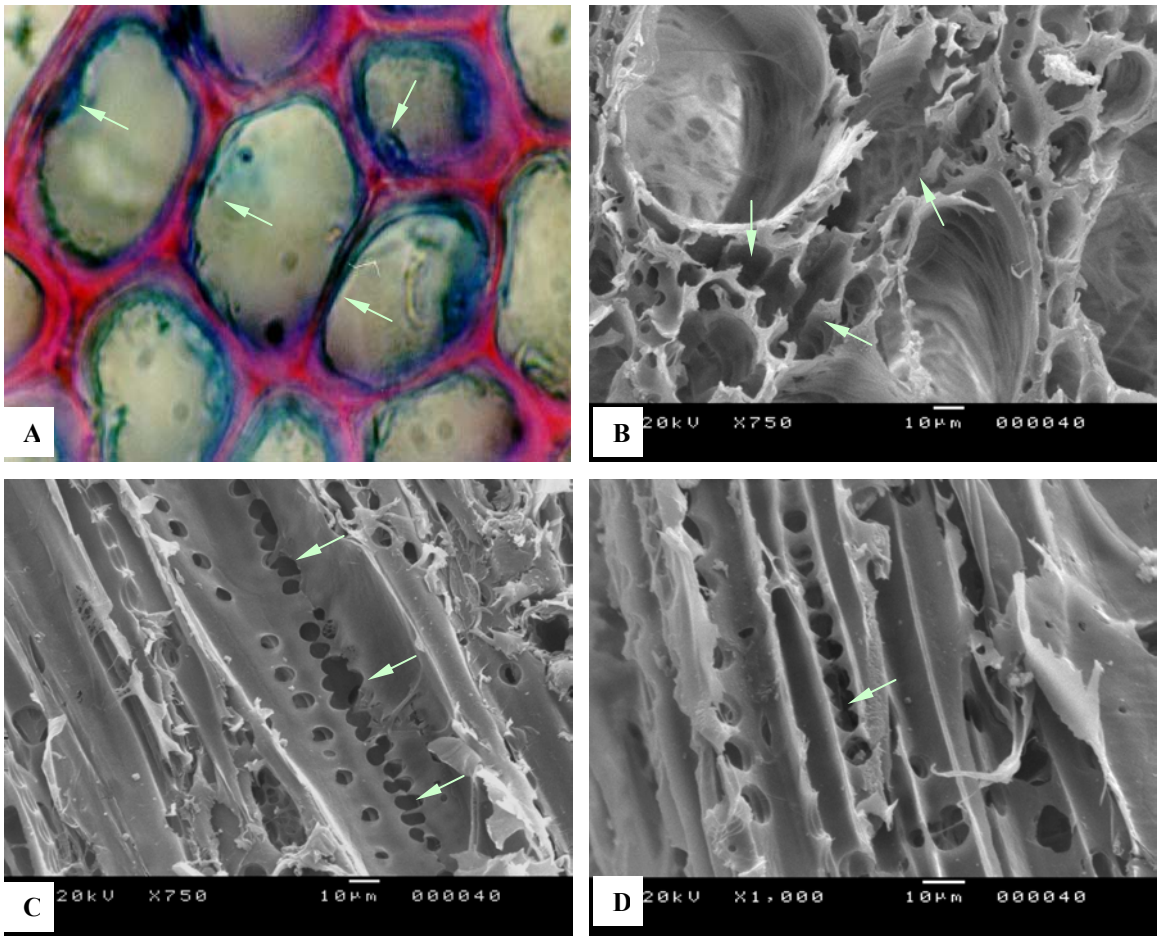


Fig. 4-1. White rot decay in non-acetylated beech. **A:** Eroding cell walls (arrows). Walls are getting thinner due to extensive erosion. Blue colour indicates cell wall modification due to delignification. **B:** Cell walls have been extensively eroded. **C:** Vessel pits (arrows) were eroded and opened due to enzymatic reactions. **D:** White rot fungus opens pits in fibbers (arrows). **A:** Cross section. **B:** Cross view; SEM.**C:** Tangential view; SEM. **D:** Radial view; SEM.

Similar stages were recognized for ray cells (fig. 4-6B). Hyphae could penetrate into the rays within the first week and decay them after 23 and 19 days in the non-acetylated wood and at WPG 3.41%. Within the second week, they could appear in rays of both moderately acetylated wood and at the highest WPG. While decay in rays begin after 19 and 46 days at WPGs 8.84% and 9.99% respectively. No decay in ray cells was distinguished at the highest WPG. Loss of birefringence in all types of wood was the same as explained above for fiber cells. Ray cells lose their birefringence in the non-acetylated wood and at weight gain 3.41% after 23 and 19 days respectively. Whereas the loss of birefringence in both moderately acetylated woods and at the highest weight gain was seen since before the first day of incubation (similar to fibers) (fig. 4-6B).

The advancement of white rot decay in the acetylated beech wood is summarized in table 4-2. It represents that the non-acetylated beech wood and the acetylated wood at the lowest WPG were severely

decayed. Slight decay was only resulted due to the moderate acetylation (WPGs 8.84% and 9.99%). At the highest WPG (17.15%), no decay was obtained. Concerning the results, “no protection” of beech wood was achieved at the lowest weight gain, while “good and better protections” were obtained at weight gains 8.84% and 9.99% respectively and “the best wood protection” was revealed at the highest weight gain (17.15%).

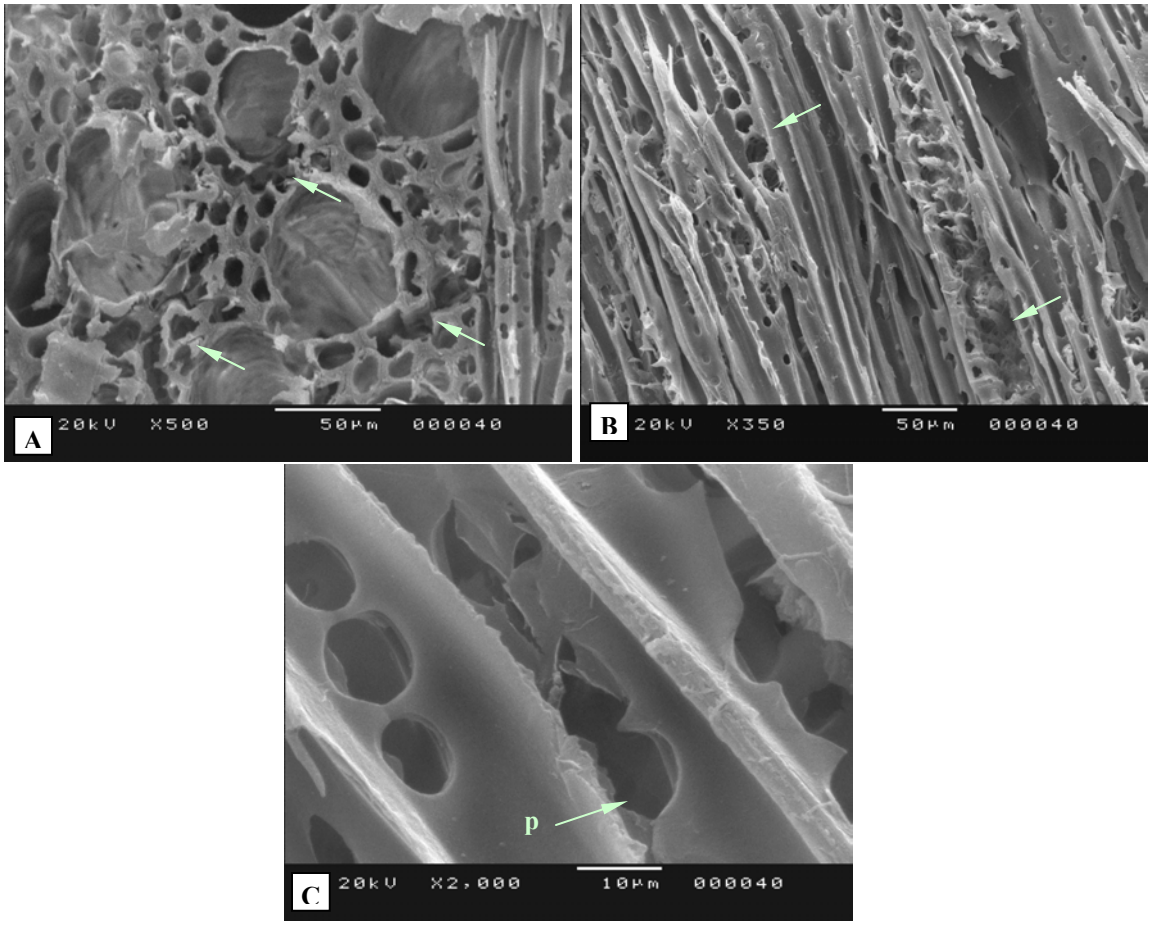


Fig. 4-2. Severe white rot decay in acetylated beech; WPG 3.41%. **A:** Severe erosion on fiber cell walls (arrows). **B:** Extensive decay in ray cells (arrows). **C:** Opened fiber pits (p) due to enzymatic activities. **A:** Cross view; SEM. **B:** Tangential view; SEM. **C:** Radial view; SEM.

Scots pine- Microscopical observations showed severe white rot decay in non-acetylated Scots pine wood (fig. 4-7). It was observed that cell walls were extensively eroded and lost their integrities due to simultaneous white rot decay (fig. 4-7 A). Pits were degraded and opened by hyphae that placed into cell lumina (fig. 4-7 B&D). Ray cells were completely dissolved by white rot fungus and their empty places were left after severe decay (fig. 4-7 C). In some cells, selective white rot decay was also observed (fig. 4-7 D). In this case, hyphae colonized in pit chambers and dissolved ML while degrading pit membranes and tori. Arrows in figure 4-7 D show decayed regions. Degradation of ML and secondary wall reveal ligninolytic and cellulolytic capabilities of *T. Versicolor*.

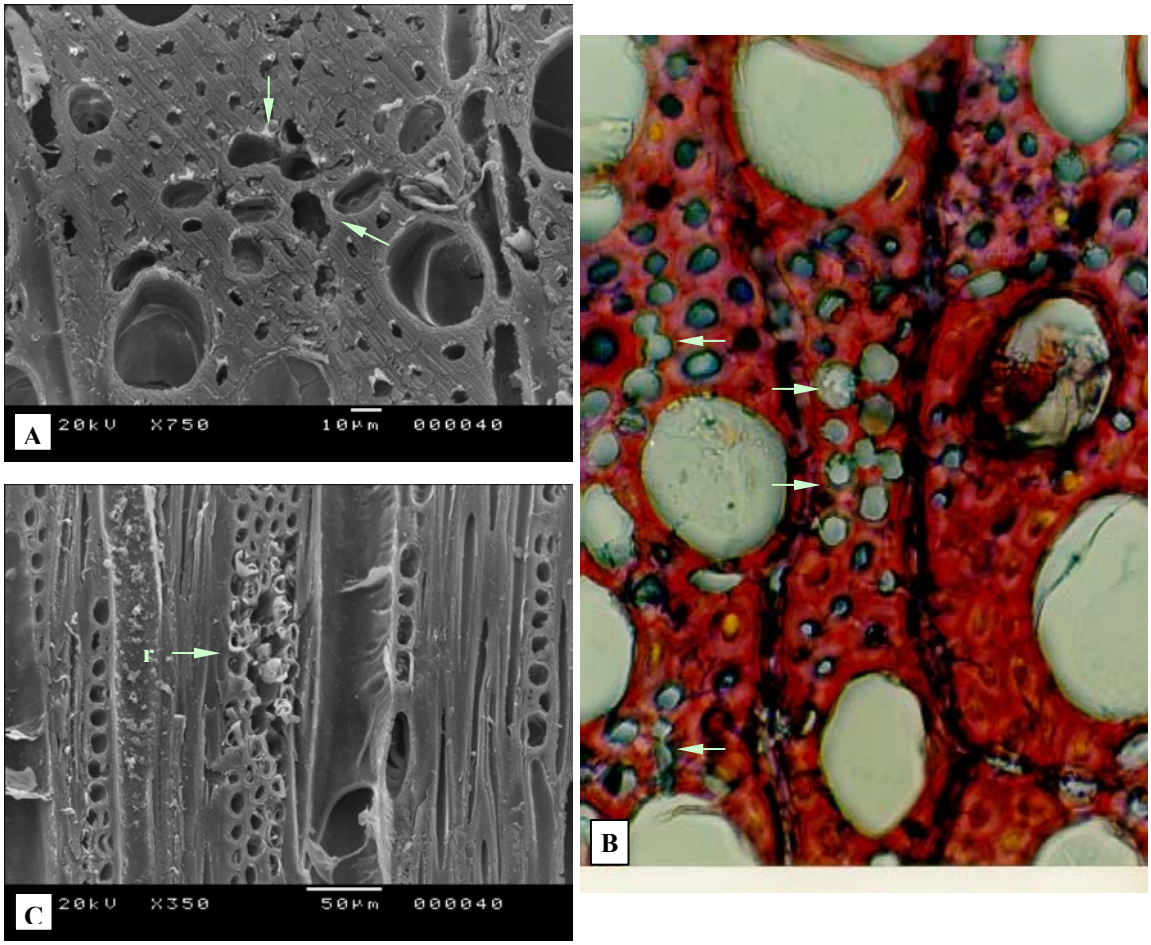


Fig. 4-3. White rot decay in acetylated beech; WPG 8.84%. **A&B:** Fiber cell walls are slowly decaying (arrows). **A:** Cross view; SEM. **B:** Cross section. **C:** Decaying ray cells (r). **C:** Tangential view; SEM.

At low degree of the acetylation (WPG 2.71%), severe decay was also observed in tracheids (fig. 4-8). It was observed that cell walls were thinning in some cells and middle lamellae were also dissolved in others (fig. 4-8 A&B). The erosion of cell walls from lumen inward ML and removing the ML indicate simultaneous white rot decay in the acetylated Scots pine wood. Ray cells were also severely attacked and empty rays were remained after degradation (fig. 4-8 C). Bordered pits were extensively decayed and opened (fig. 4-8 D). These evidences show advanced stage of the decay at low degree of acetylation in Scots pine wood.

In moderately acetylated pine wood (WPG 9.97%), white rot decay was also distinguished (fig. 4-9). Tracheid walls were eroded and thinned scarcely, especially around resin canals (fig. 4-9 A). After severe attack in ray cells, rays were remained empty (fig. 4-9 B). Hyphae placed in pit chambers and removed pit borders and opened the pits by dissolving them (fig. 4-9 C). At WPG 10.97%, scarce white rot decay was also observed, especially in tracheids surrounding resin canals (fig. 4-10 A). However, severe attack was observed in rays (fig. 4-10 B). Hyphae colonized in pits and opened them by dissolving the pit borders and tori (fig. 4-10 C) by producing bore holes in the tori (fig. 4-10 D). The evidences indicate early stage of white rot decay at WPG 10.97%.

At the highest weight gain (WPG 19.95%), no considerable decay patterns were distinguished (fig. 4-11). However it was observed that hyphae colonized into rays and degraded slightly the ray cells (fig. 4-11 B&C). Hyphae use the rays to distribute in wood structure and penetrate into other cells (fig. 4-11 C). Fungal colonization was also observed in pits, while no decay was distinguished (fig. 4-11 D).

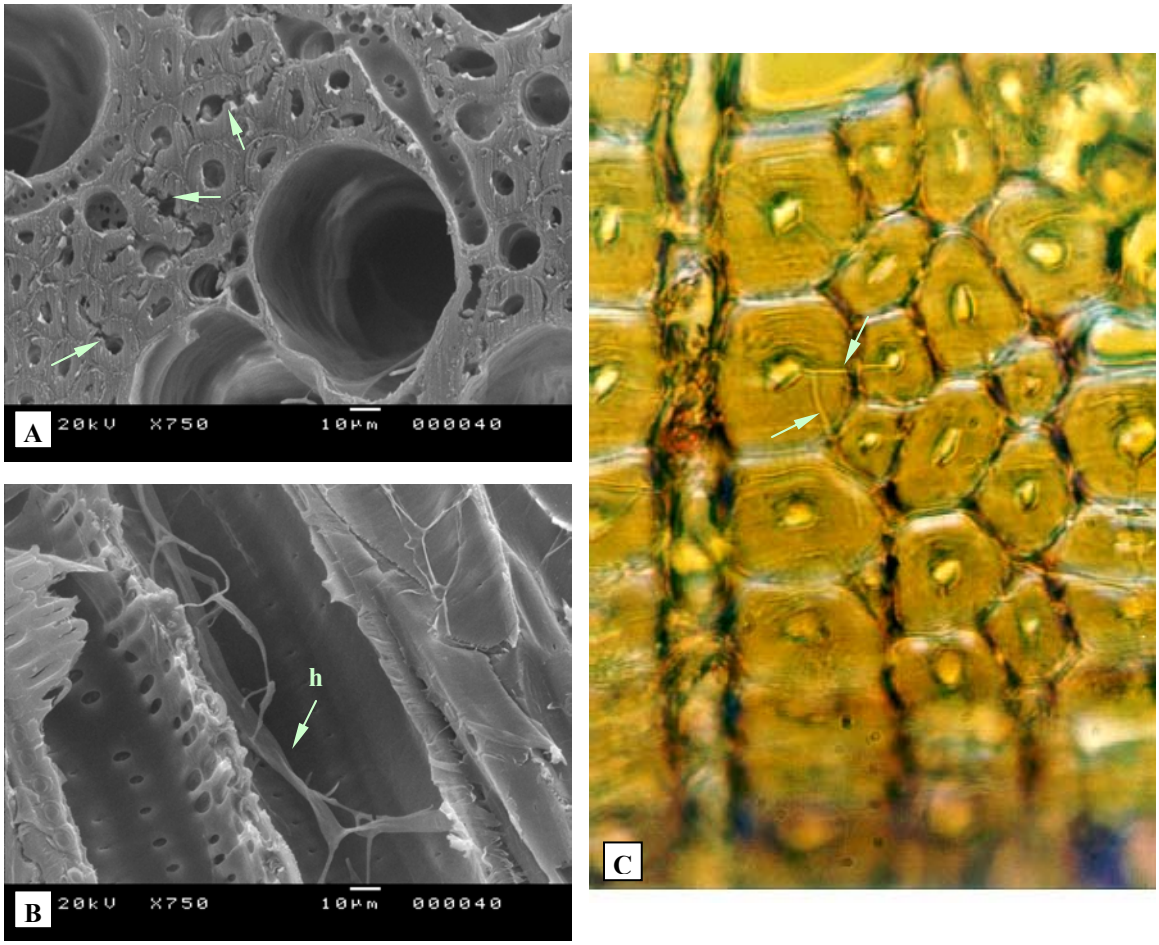


Fig. 4-4. White rot decay in moderately acetylated beech; WPG 9.99%. **A:** Slight decay in fiber walls. **B**: Hyphae exist in cell lumina and colonize there to decay wood. **C:** White rot hyphae penetrate through the fiber walls (arrows); Nomarsky. **A:** Cross view; SEM. **B:** Radial view; SEM. **C:** Cross section.

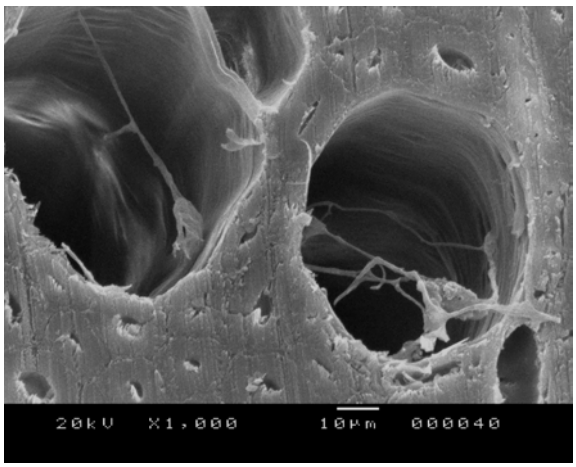


Fig. 4-5. No white rot decay in highly acetylated beech wood; WPG 17.15%. Hyphae (h) could colonize into vessel lumina, while they cannot decay highly cell walls. Cross view; SEM.

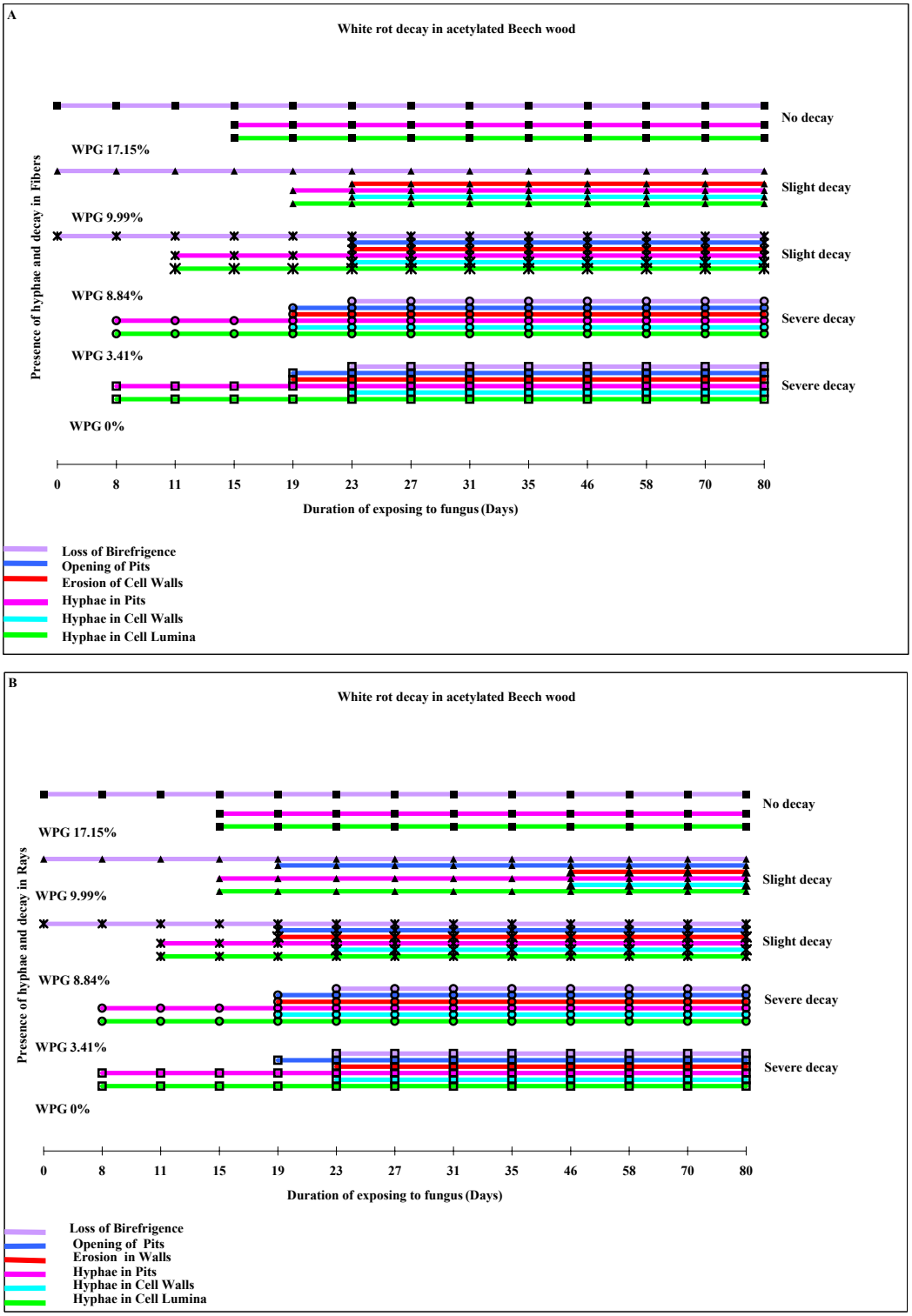


Fig. 4-6. Advancement of white rot decay (*Trametes versicolor*) in acetylated beech wood; **A:** Fibers; **B:** Rays

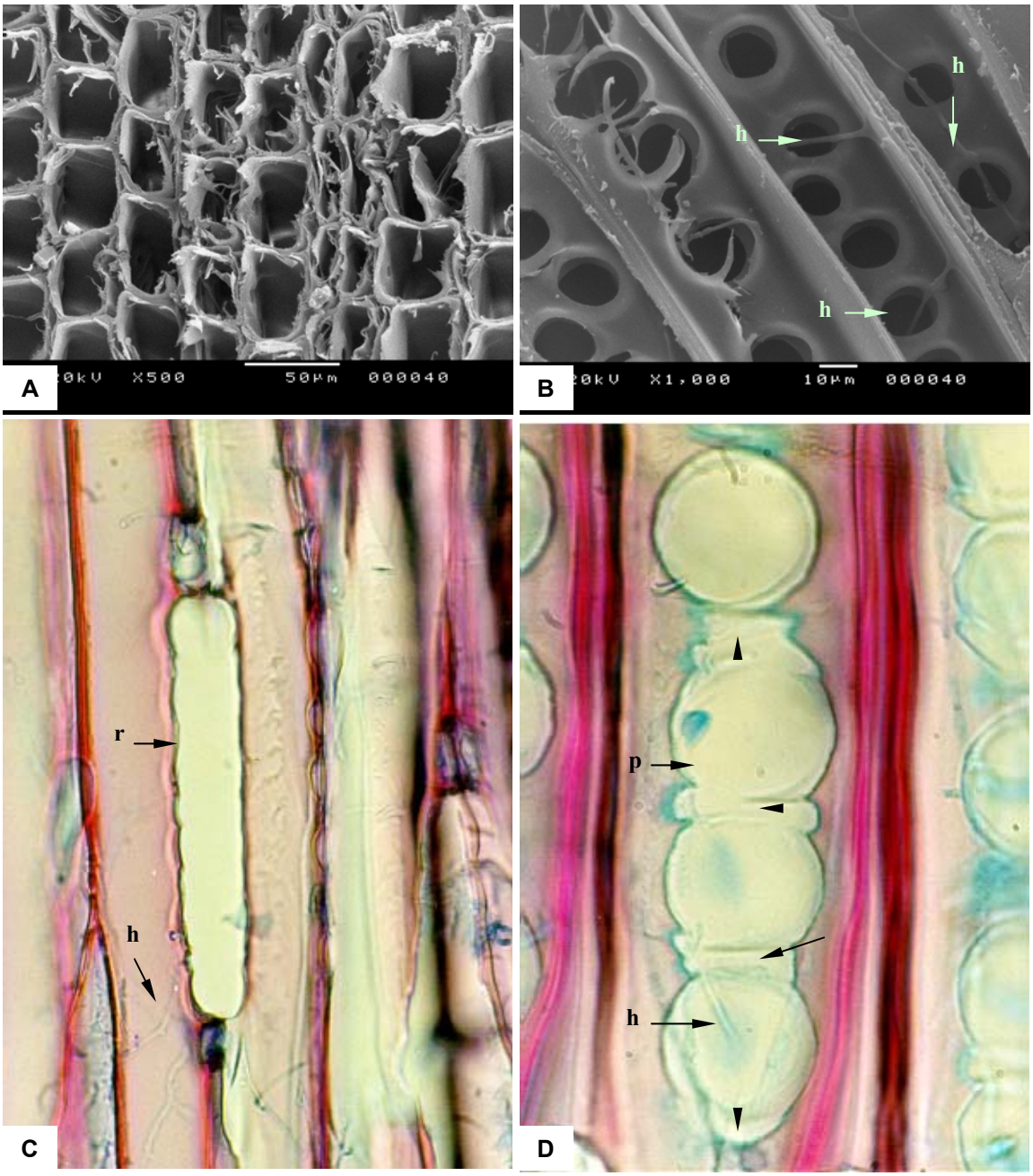


Fig. 4-7. Severe white rot decay in non-acetylated Scots pine wood. **A:** Cell walls are extensively decaying. Cross view; SEM. **B:** Tracheid pits were severely decayed by white rot hyphae (h). Tangential view; SEM. **C:** White rot fungus erodes ray cells. Ray cells were removed and their empty places were left after decay; Tangential section. **D:** Hyphae (h) dissolve pit borders (p) and tracheid wall and ML (arrow heads) leave nothing after severe erosion; Radial section

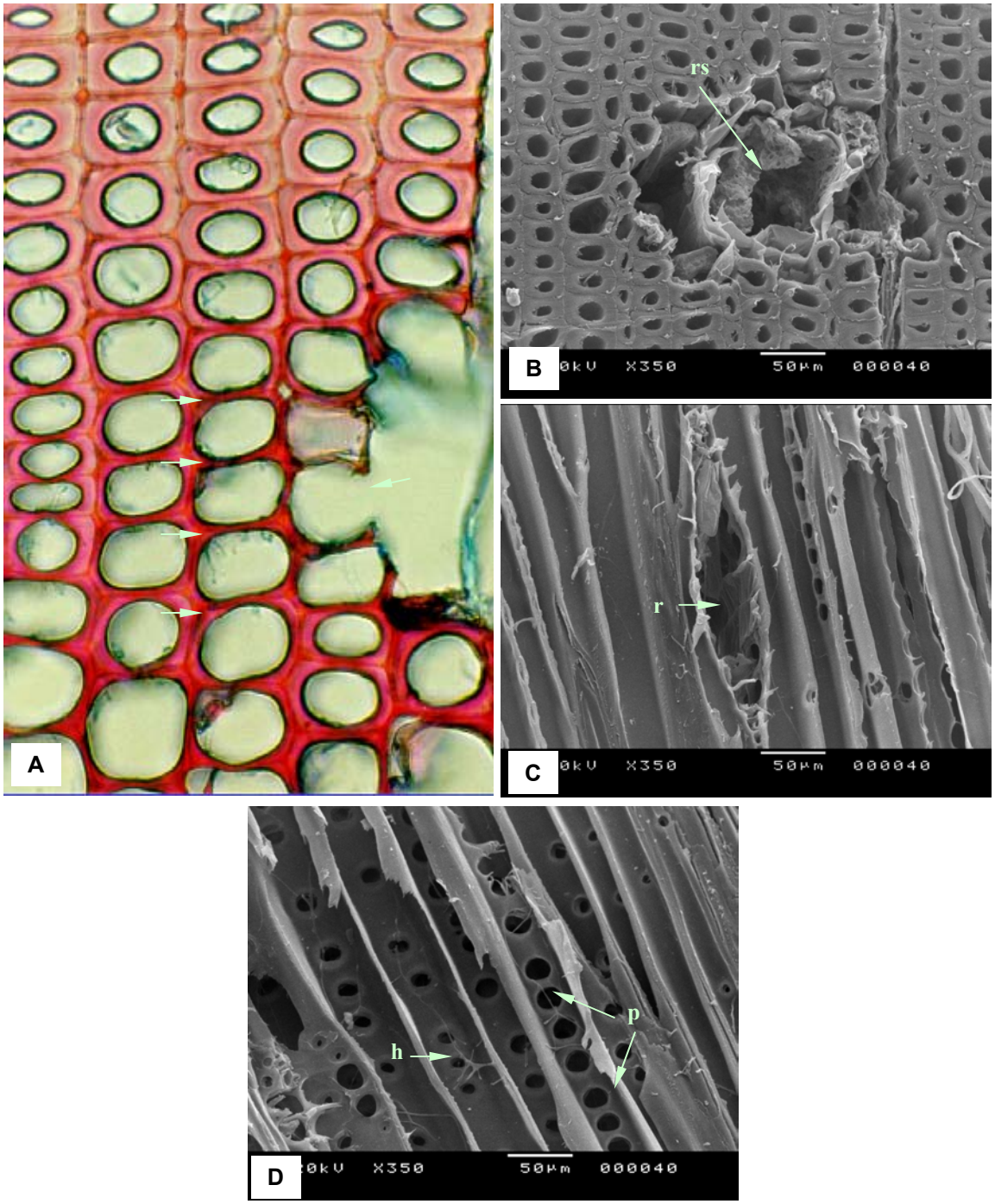


Fig. 4-8. Severe white rot decay in acetylated Scots pine; WPG 2.71%. **A:** White rot fungus erodes cell walls from luminal side inward ML by producing their enzymes (arrows) and leaves broken cells. **B:** A severely decaying resin canal (rs). Eroding tracheid cell walls around resin canal are seen. **C:** Empty rays (r) remain after severe white rot decay. **D:** Hyphae (h) open the pits (p) by dissolving their borders. **A:** Cross section. **B:** Cross view; SEM. **C:** Tangential view; SEM. **D:** Radial view; SEM.

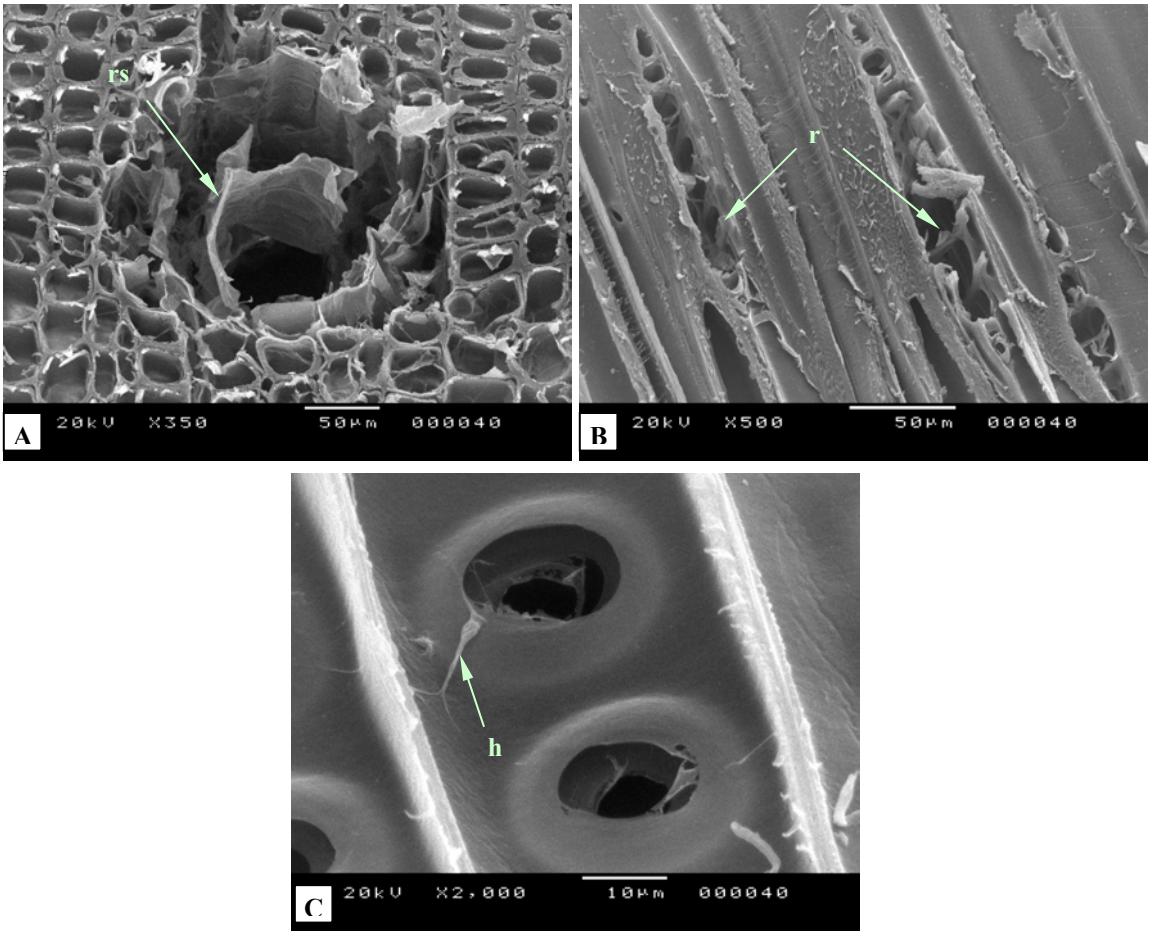


Fig. 4-9. White rot decay in moderately acetylated Scots pine; WPG 9.97%. **A:** Cells are eroding by white rot fungus. Resin canal (rs) is also decayed by eroding hyphae. **B:** White rot fungus erodes ray cells and leaves empty rays (r) after severe decay. **C:** Hyphae (h) erode pit borders to open them for colonizing into pit chambers to access to ML. **A:** Cross view; SEM. **B:** Tangential view; SEM. **C:** Radial view; SEM.

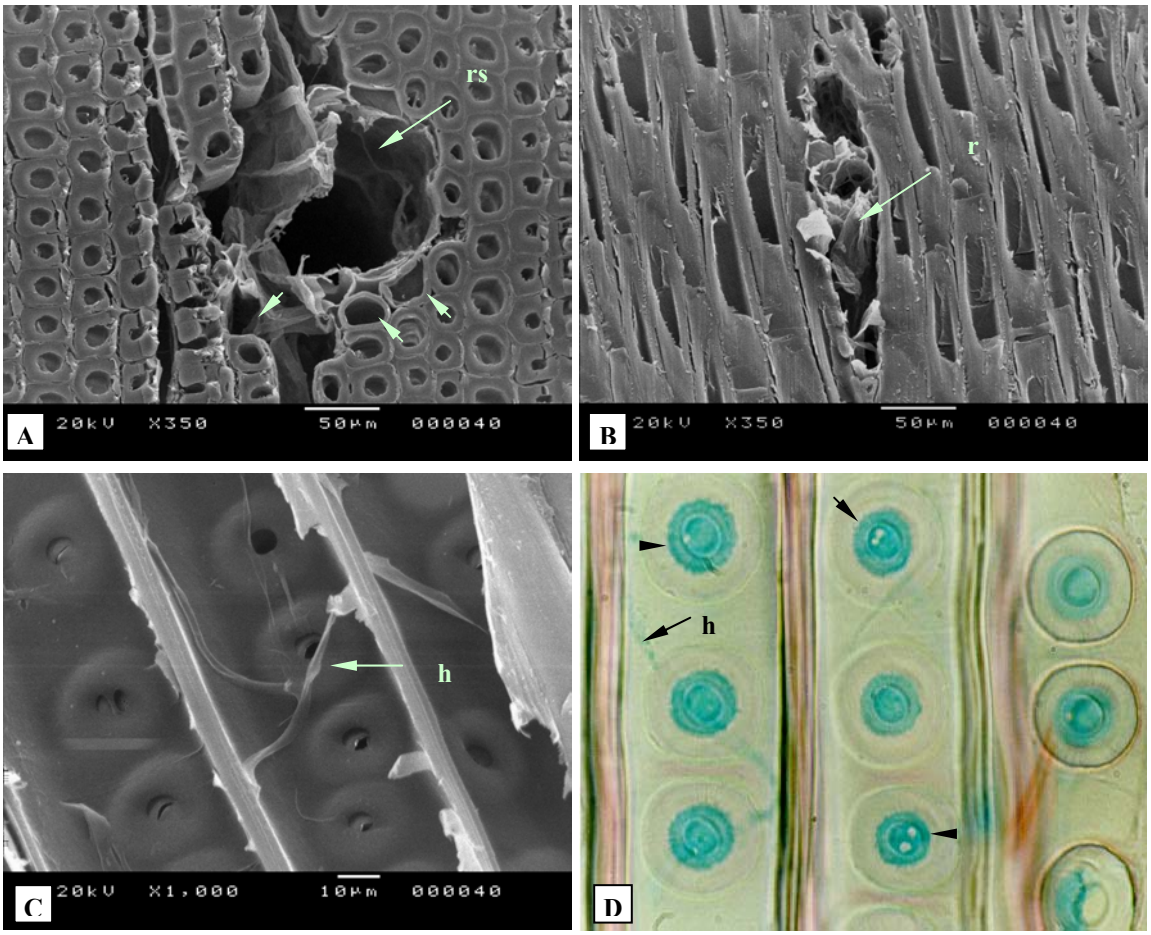


Fig. 4-10. White rot decay in moderately acetylated Scots pine; WPG 10.97%. **A:** Severe decay in resin canals (rs) and erosion in surrounding cell walls (arrow heads). Cross view; SEM. **B:** Ray cells (r) are decaying extensively. Tangential; SEM. **C&D:** Hyphae (h) are colonizing into pit chambers and eroding pit membranes. Blue color in pit chambers indicates the enzymatic delignification on tori. Bore holes are seen in tori (arrow heads). **C:** Radial view; SEM. **D:** Radial section.

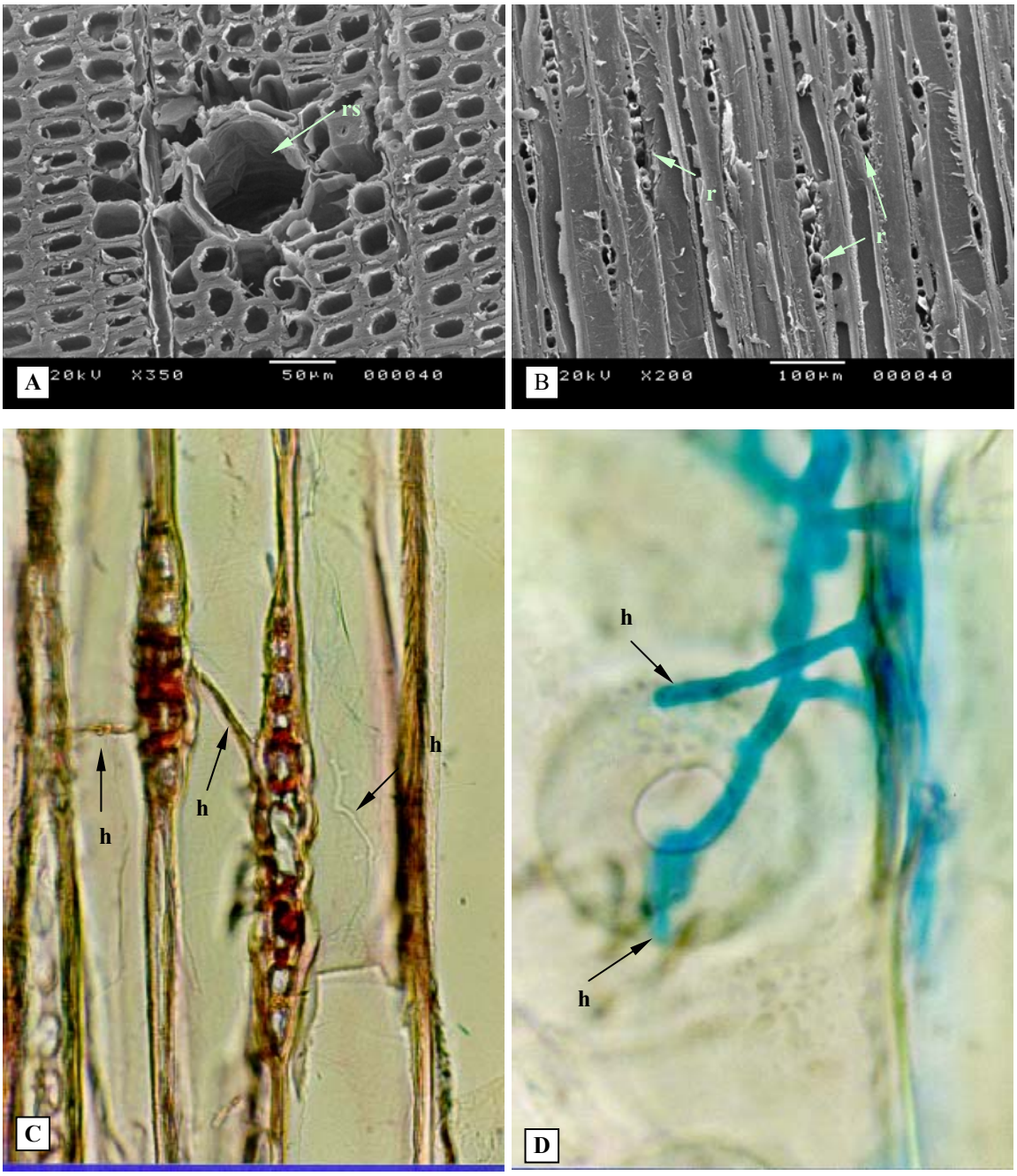
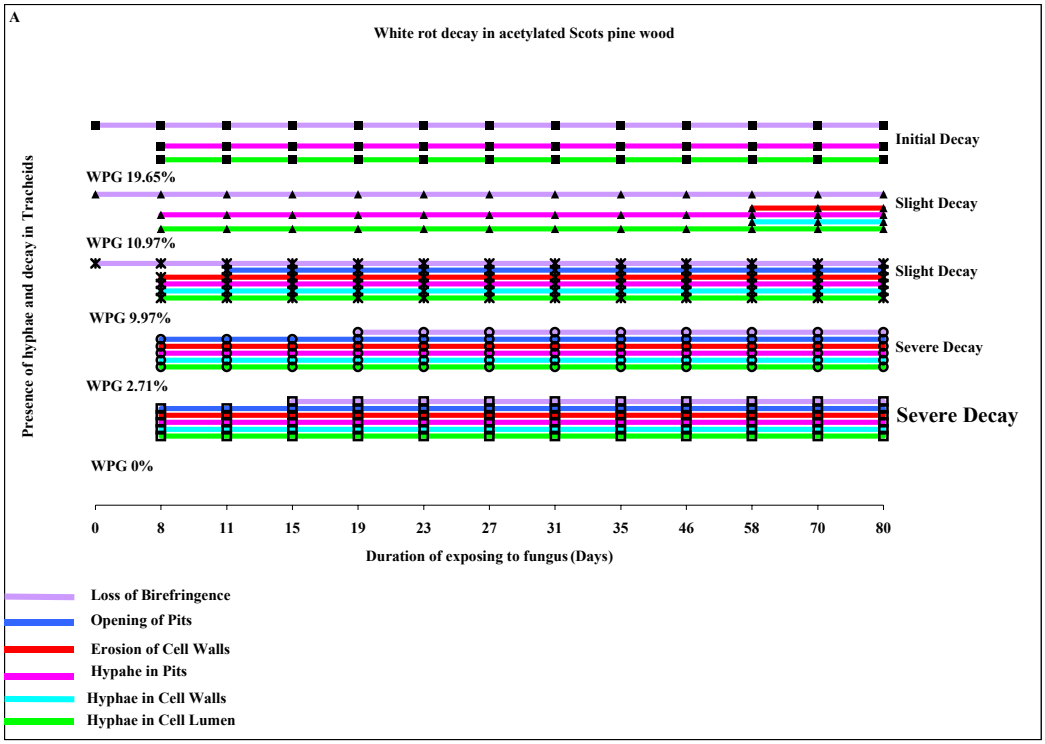


Fig. 4-11. White rot decay in highly acetylated wood; 19.95%. A: No decy in traheid cell walls. Resin canal (rs) is seen intact. Cross view; SEM. B&C: Hyphae (h) colonize into rays (r) and intial decay occurs in the cells. B: Tangential view; SEM. C: Tangential section. D: Fungal hayphae (h) colinize into pit cahmbers to decay pit borders and ML. However no decay still occurs in the pits; Radial section.



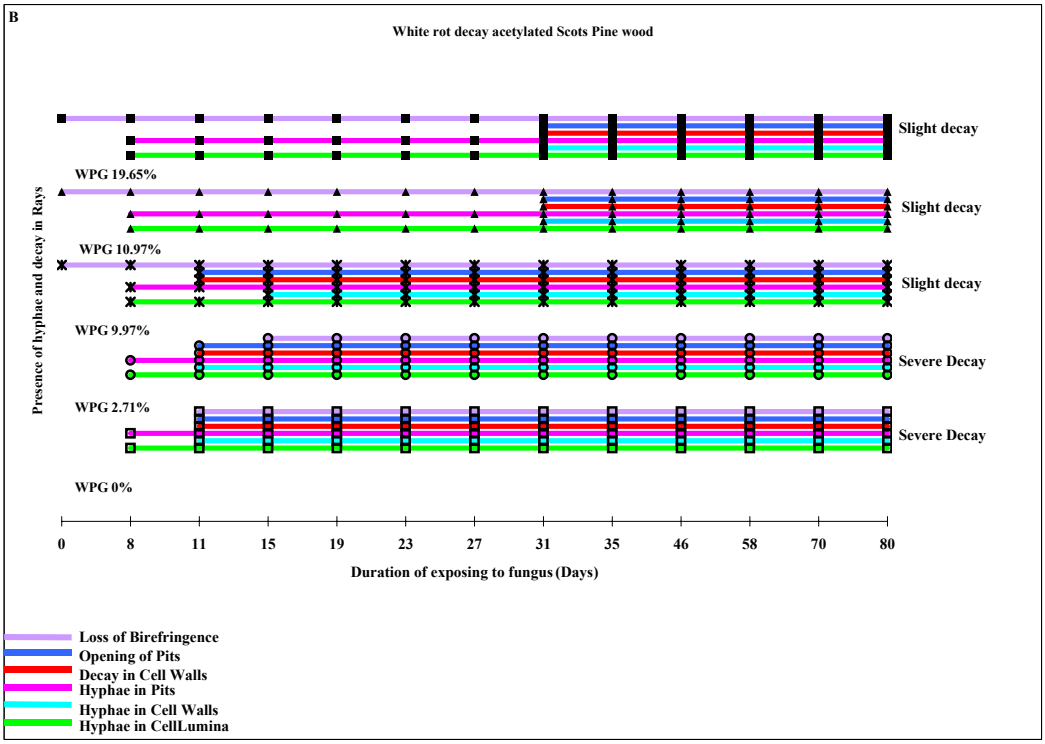


Fig. 4-12. Advancement of white rot decay (*Trametes versicolor*) in acetylated Scots pine wood

Phenology of white rot decay in non-acetylated and acetylated pine wood is shown in figure 4-12. It represents that hyphae colonized into tracheid cell lumina of acetylated and non-acetylated wood within the first week of incubation (fig. 4-12A). Opening of the pits and erosion of the cell walls have been begun in non-acetylated wood and at weight gains 2.71% and 9.97% since the first week. However, decay was advanced in the non-acetylated wood and the lowest weight gain, while cells were eroded slightly at WPG 9.97%. Erosion of cell walls has started after 58 days of incubation at weight gain 10.97%, while no erosion has still begun at the highest weight gain (19.65%). Colonization of hyphae in rays has seen since the first week of the incubation in all types of wood (fig. 4-12B). Opening of the pits and erosion of the cell walls have occurred in the non-acetylated pine wood and at WPGs 2.71% and 9.97% after 11 days of incubation period. Erosion of the rays and opening of their pits were distinguished after 31 days in both weight gains 10.97% and 19.65%. Rays were severely decayed in the non-acetylated wood and at the lowest weight gain. However, it was slightly decayed in the moderately acetylated wood and the highest weight gain. Polarized microcopy revealed that wood lose its birefringence at the lowest weight gain and the non-acetylated wood due to white rot decay, while loss of the birefringence was observed in unexposed samples of acetylated pine at above 9.97% of weight gains (fig. 4-12A&B). This reveals also impractical usage of polarized microscopy for acetylated pine wood.

Table 4-2 represents the advancement of white rot decay in acetylated pine wood. Concerning the summary of results, severe white rot decay was distinguished in non-acetylated pine and the lowest weight gains and consequently the acetylation protection level was categorized at “no protection”. Slight/scarce white rot decay in both moderately acetylated wood (WPGs 9.97% and 10.97%) placed the acetylation in “good/better protection” level. And finally due to initial stage of white rot decay, “well protection” was considered for the highest weight gain (19.65%) due to scarce ray decay that was distinguished at weight gain 19.65%.

Colonization of white rot fungal hyphae in fibers and tracheids cell lumina in the non-acetylated and the acetylated woods reveals the capability of the white rot fungus to access to all cells even the innermost cells. However, they can only severely erode cell walls in the non-acetylated and the acetylated wood with the lowest weight gains. Penetrated hyphae into fibers and tracheids lumina could scarcely attack cell walls in moderately acetylated wood and have no capability to erode highly acetylated fiber cell walls. These evidences reveal that acetylation increase the durability of wood and protects it against the white rot fungus *Trametes versicolor* when the degree of the acetylation in wood raise above 8.84% in beech and 9.97% in pine. The wood is very well protected when it is acetylated at higher weight gains, e.g. 17.15% and 19.65%. Overall observations showed that white rot attack was suppressed above about 10% of weight gains and full protection could be achieved at higher than about 20% of weight gains. The observation will be discussed in details.

4.3.2. *Poria placenta*

Beech- Severe brown rot decay was distinguished in the non-acetylated beech (fig. 4-13). Broken and collapsed cell walls indicate advanced stage of brown rot decay (fig. 4-13 A-C). Cells have lost their integrities and separated from each other due to dissolved middle lamellae (fig. 4-13 C). This evidence (dissolved ML) revealed ligninolytic activity of *Poria placenta*. Rays cells were influenced by the fungus and separated from each other. And their cells seemed broken (fig. 4-13 D&E). Hyphae produced bore holes on cell walls to penetrate into neighboring cells (fig. 4-13 F). Using mixture of safranin-astra blue showed some cells have blue color after severe brown rot attack (fig. 4-13 B). It indicates ligninolytic activity in brown rot fungus, because polysaccharides remained after delignification process in some cells.

At low degree of the acetylation (WPG 3.41%), extensive brown rot decay was also observed in the acetylated beech wood (fig. 4-14). Collapsed and broken cell walls were distinguished (fig. 4-14 A&B). Cells were seen separated and in some cases ML were dissolved due to enzymatic activities (fig. 4-12 C). Porous structures were also observed in fiber cell walls (fig. 4-14 C). Eroded cell walls, removed ML and porous secondary cell wall layers indicate cellulolytic and ligninolytic activities in brown rot fungus *P. placenta*. It was also observed that highly lignified ray cells were attacked by fungus (fig. 4-14 D&E). Ray cells have separated due to attacked ML.

At WPG 8.84% in acetylated beech wood, brown rot decay was also distinguished (fig. 4-15). The decay was not so severe as the non-acetylated wood or the lowest WPG (3.41%). It was observed that S_2 layer in fiber cell walls have been modified and their color changed to blue (fig. 4-15 B&C). The blue color

indicates that lignin has been removed from S_2 layers and polysaccharides were remained. However S_1 layer and compound middle lamella in fiber cell walls have their red color, indicating the presence of lignin in those cell wall layers. It reveals that lignin still has not been attacked. Microscopical observation revealed that ray cells were intact and hyphae could not decay those cells (fig. 4-15 D). In moderately acetylated beech wood at WPG 9.99%, slight brown rot decay was distinguished (fig. 4-16). However, scarce pores were in S_2 layers (fig. 4-16 A). It indicates that polysaccharides are removing from cell walls. Anyhow, the removal is not so effective. It was observed that hyphae produce bore holes in cell walls to penetrate into the other cells to find accessible source of nutrients (fig. 4-16 B-D). Delignification process was also observed in some cells (fig. 4-16 B). As it was explained above, this evidence indicates that polysaccharides were remained after delignification process. Producing bore holes on highly lignified vessel walls also indicate capability of brown rot fungus to degrade lignin (fig. 4-16 C). Ray cells were observed with no decay and they were remained intact after incubation period (fig. 4-16 E).

At the highest WPG (17.15%), colonization of hyphae was observed in cell lumina, especially in vessels (fig. 4-17 A). And they could produce slight brown rot decay in highly acetylated fiber walls (fig. 4-17 B). In the acetylated fiber cell walls, it was observed that red color of the S_2 layer was changing into pale bluish color (fig. 4-17 C). This phenomenon proves initial removal of lignin before polysaccharides. No brown rot attack was observed in ray cells and they were seen intact (fig. 4-17 D). Microscopical investigations revealed that hyphae could produce bore holes on highly acetylated fiber cell walls (fig. 4-17 E) and also vessels (fig. 4-17 F).

The phenology of brown rot attack according to the time indication of decay stages in acetylated beech wood is represented in figure 4-18. Concerning the results, colonization of brown rot hyphae in fiber cell lumina occurred at same time in all types of wood after 11 days of incubation (fig. 4-18A). However decay revealed in fiber cell walls of non-acetylated wood and low level of the acetylation (WPG 3.41%) after 11 days when the colonization of hyphae occurred, while decay distinguished at weight gain 8.84% after 23 days of exposure to fungus and it was occurred after 35 days in moderately acetylated beech wood (WPG 9.99%). At the highest weight gain, decay slowly began and hyphae degrade highly acetylated fibers slightly after 58 days of incubation. Fungus could produce bore holes since day 11 in non-acetylated, low and moderately acetylated woods, while their formation occurred after 23 days of incubation at WPG 17.15%. Erosion of pits and formation of bore holes on pits were distinguished after 11 days in non-acetylated beech wood and weight gains 3.41% and 8.84%. The appearance of bore holes was observed 35 and 46 days later at weight gains 9.99% and 17.15% respectively. After 31 days of the incubation, the non-acetylated wood and the wood with 3.41% of the acetylation were severely decayed and the wood lost its integrity. However above weight gain 8.84%, acetylated woods were decaying slightly. Loss of birefringence in the non-acetylated beech wood and the lowest weight gain by using polarized light was observed at same time of colonization and erosion of fiber cell walls (day 11). This evidence reveals aggressiveness of brown rot fungus and immediate attack of crystalline cellulose. Under polarized light, it was also observed that the acetylated sound beech wood samples above weight gain 8.84% have lost their birefringence properties before any exposure to brown rot fungus.

Figure 4-18B also shows advancement of brown rot decay in rays of the acetylated beech wood. Colonization of hyphae were observed in rays of the non-acetylated wood and the lowest weight gain (3.41%) within the first week of incubation, while it began after 11 days in the acetylated wood at weight gains above 8.84%. Erosion of ray cell walls in the non-acetylated wood began simultaneously after 8 days of the colonization. At the lowest weight gain, visible erosion of rays began lately after 19 days, while it was few days earlier at weight gain 8.84%. No brown rot decay was revealed in ray cell walls of the moderately acetylated wood (WPG 9.99%) and the highest weight gain (17.15%). Opening of ray pits occurred at same time of hyphal colonization in the non-acetylated wood and low and moderate weight gains (3.41% and 8.84%). At WPG 8.84% openings were observed after 11 days. No opening of ray pits were distinguished at weight gains 9.97% and 17.15%. Polarized microscopy showed loss of birefringence in the non-acetylated wood and at the weight gain 3.41% after 11 days of incubation. While ray cells of intact acetylated wood above 8.84% of weight gains lost their birefringence before exposing to fungus. This evidence indicates also disadvantage of polarized microscopy in the acetylated wood (This fact will be explained later). Due to aggressive attack by brown rot fungus in the non-acetylated wood and the lowest WPG, majority of ray cells were decayed extensively and lost their integrities after 31 days. However above WPG 8.84%, rays were attacked slightly.

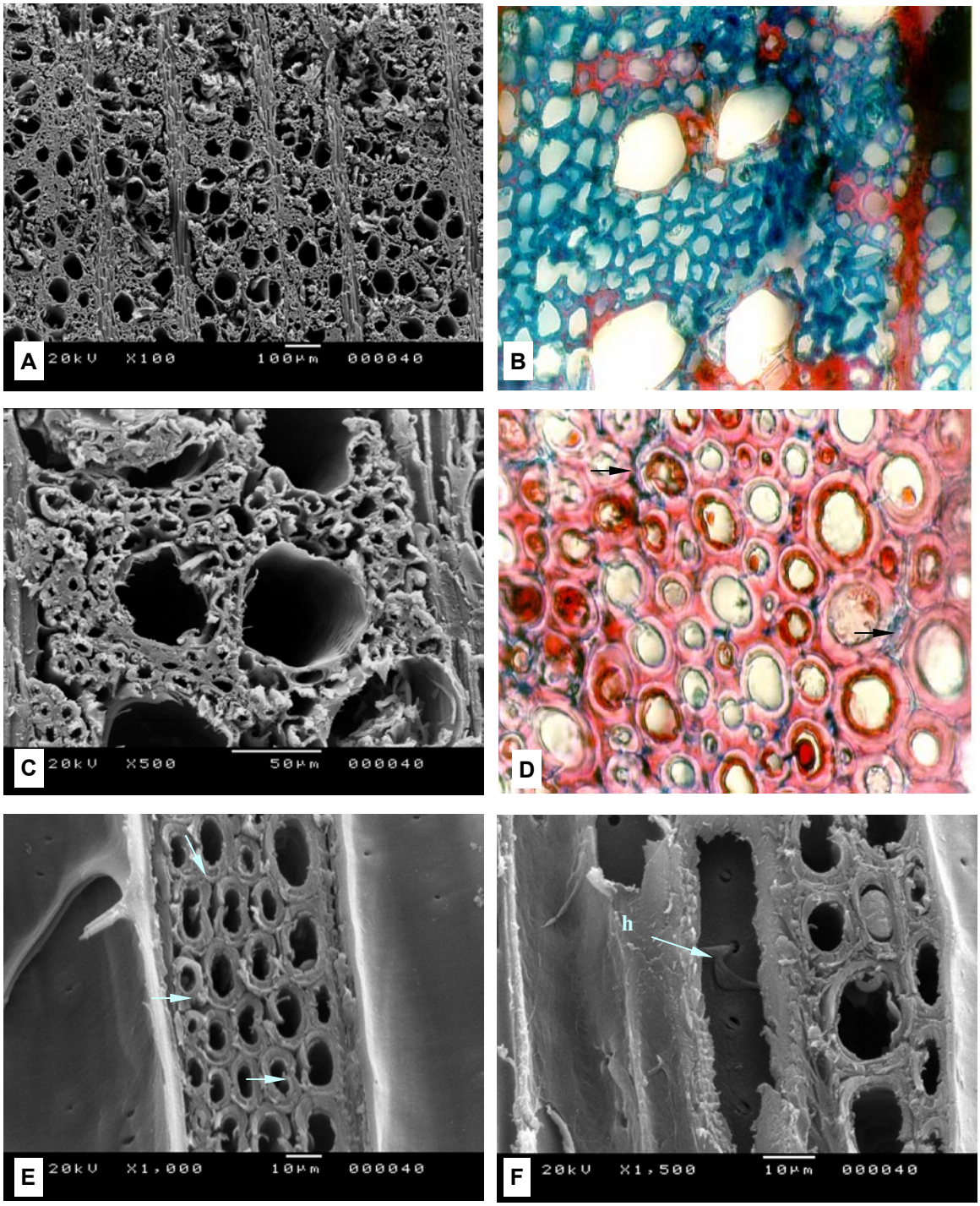


Fig. 4-13. Severe brown rot decay in non-acetylated beech wood. **A-C:** Advanced stage of brown rot decay in cells. Cell walls have collapsed and separated due to extensive degradation. **A:** Cells do not have their typical figures. **B:** Broken and collapsed cells show the heavy attack of fungus. Blue color indicates a delignification process in the cell walls. Lignin has been removed, however polysaccharides were remained. This indicates a lignolytic activity of brown rot fungus. **C:** Separated cells reveal removal of ML. **D-E:** Ray cells are being separated due to heavy attack (arrows). **F:** Hypha makes bore hole on the cell wall after penetration from ray cells. **A&C:** Cross view; SEM. **B:** Cross section. **D:** Tangential section. **E&F:** Tangential view; SEM.

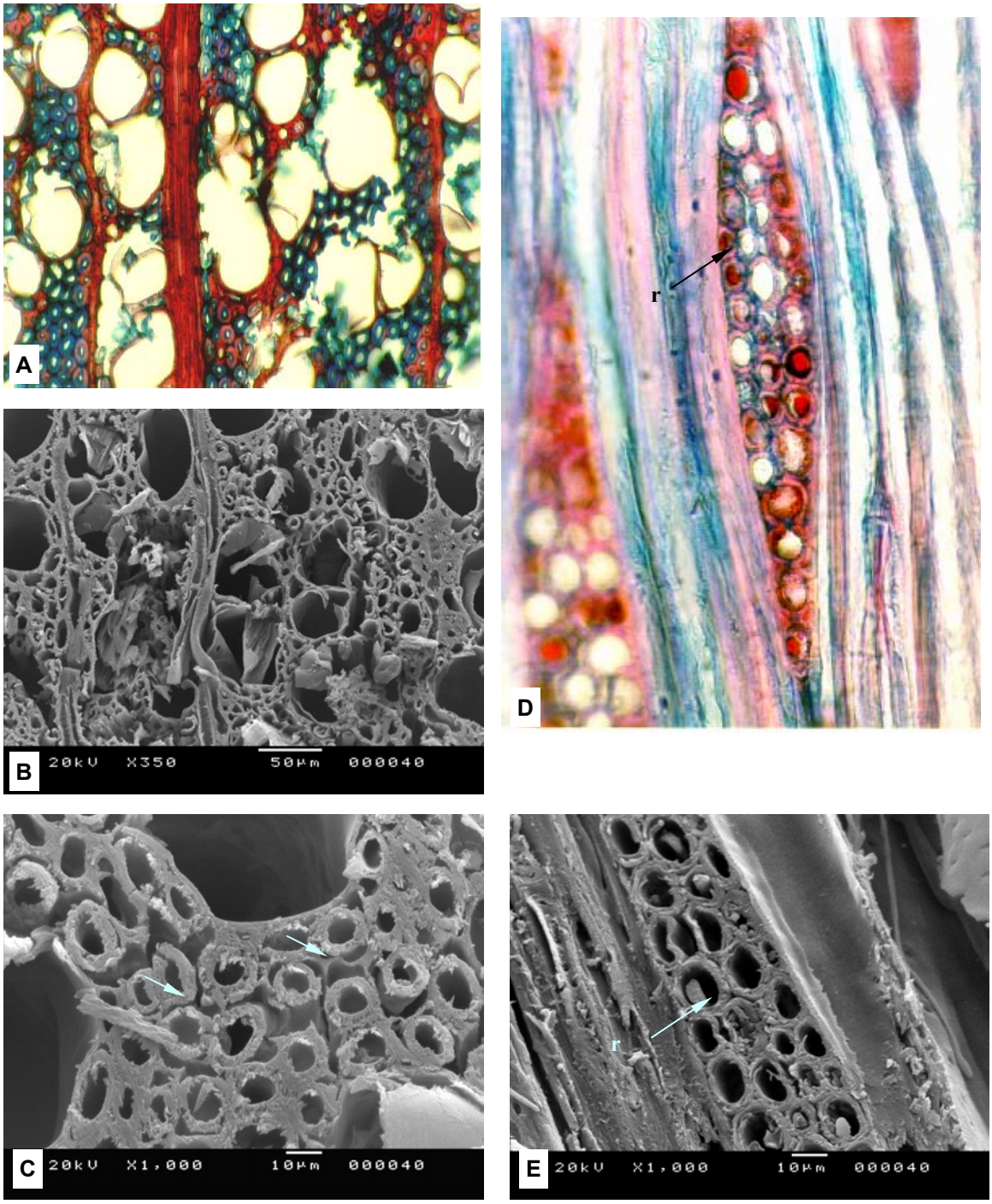


Fig. 4-14. Severe brown rot decay in acetylated beech; WPG 3.41%. **A&B:** Broken and collapsed cell walls indicate an extensive attack. **A:** Blue color reveals a lignolytic activity of fungus and eroded cells reveal cellulolytic activities of the fungus. **B:** Cells have been collapsed and lost their integrities. **C:** Removing or removed ML (arrows) indicate lignolytic activity, while porous appearance of S_2 layer of fiber cells indicates removal of polysaccharides. **D&E:** Highly lignified ray cells (r) are being degraded and separating from each other. **A:** Cross section. **B&C:** Cross view; SEM. **D:** Tangential section. **E:** Tangential view; SEM.

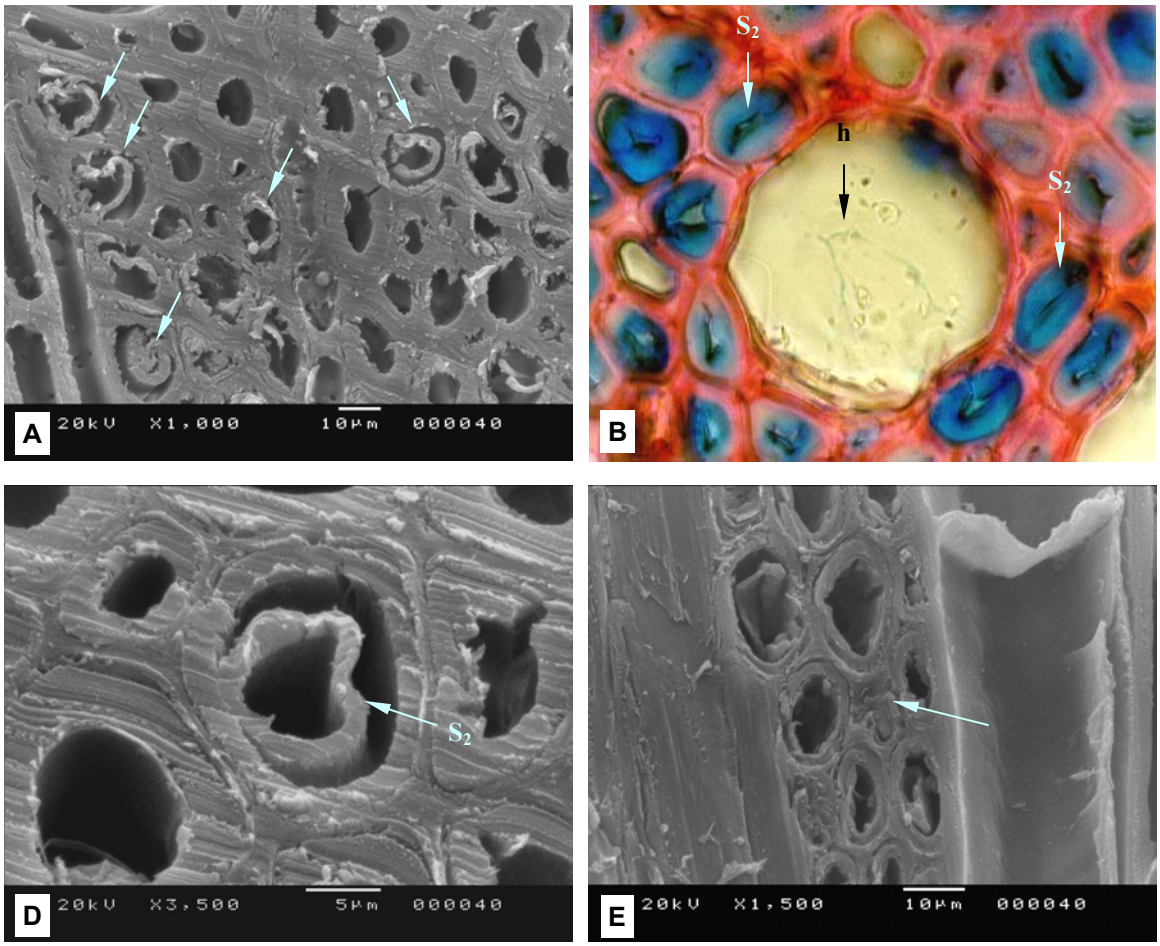


Fig. 4-15. Brown rot decay in acetylated beech; WPG 8.84%. **A-C:** Slight decay in fibers indicates low protection of acetylation at WPG 8.84%. Fiber cell walls (S_2) are modified due to enzymatic activities. **A&C:** Modified S_2 layers (arrows) have been detached during SEM preparation procedure due to weak strength. **B:** Modifying S_2 layer in fibers has blue color. This indicates still polysaccharides are remaining in cell walls due to fungal delignification process. Red color indicates that lignin still presents into cell walls. **D:** Ray cells (arrow) still are intact. **A&C:** Cross view; SEM. **B:** Cross section. **D:** Tangential view; SEM.

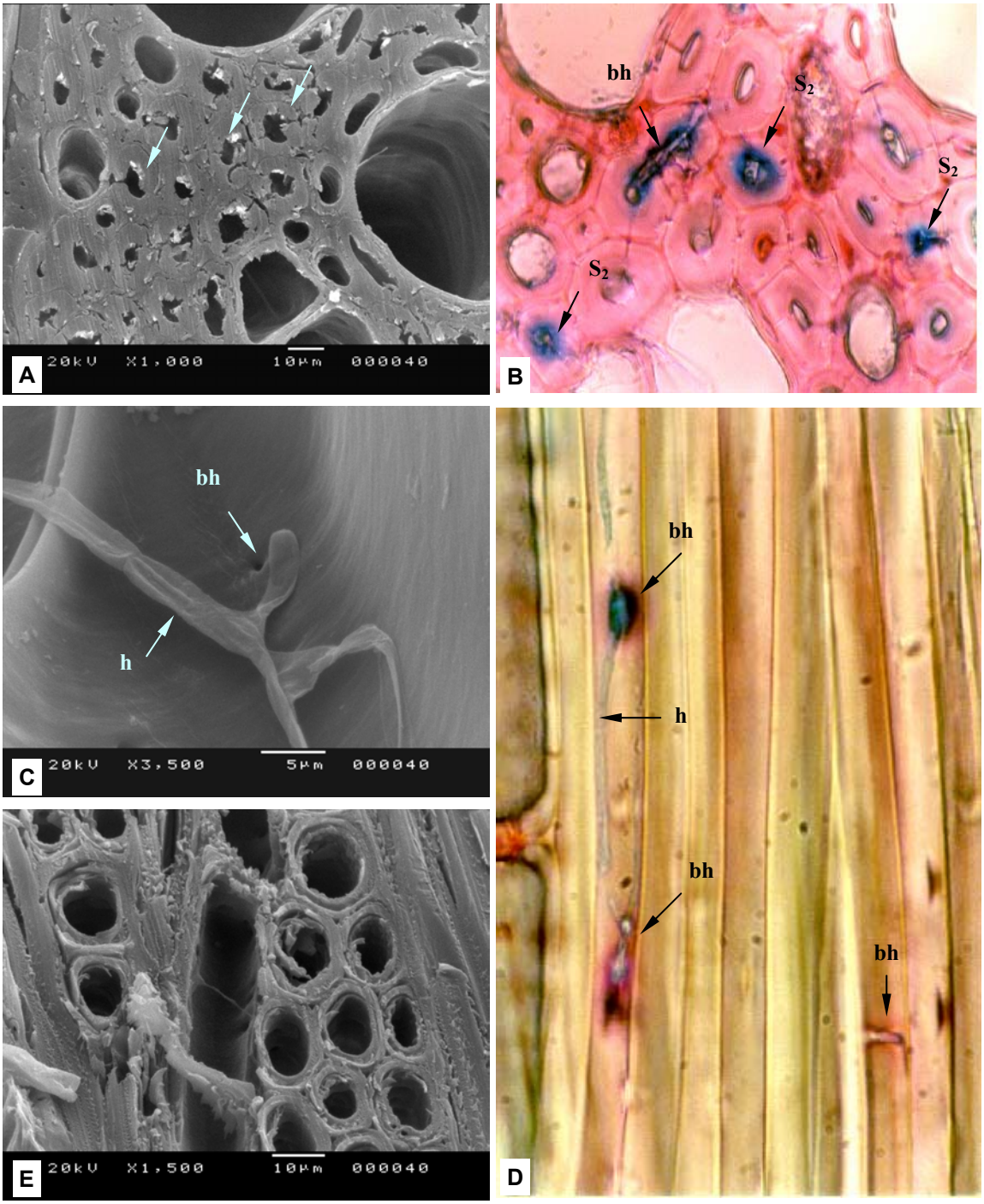


Fig. 4-16. Brown rot decay in acetylated beech; WPG 9.99%. **A:** Slight brown rot decay in fibers (arrows). Porosity in secondary wall layers indicates enzymatic activities. **B:** Hyphae produce bore holes (bh) and delignify S_2 layer in fibers. **C&D:** Hyphae (h) produce bore holes (bh) in vessels and fibers. **E:** Ray cells are still intact. **A:** Cross view; SEM. **B:** Cross section. **C:** Radial view; SEM. **D:** Radial section. **E:** Tangential view; SEM.

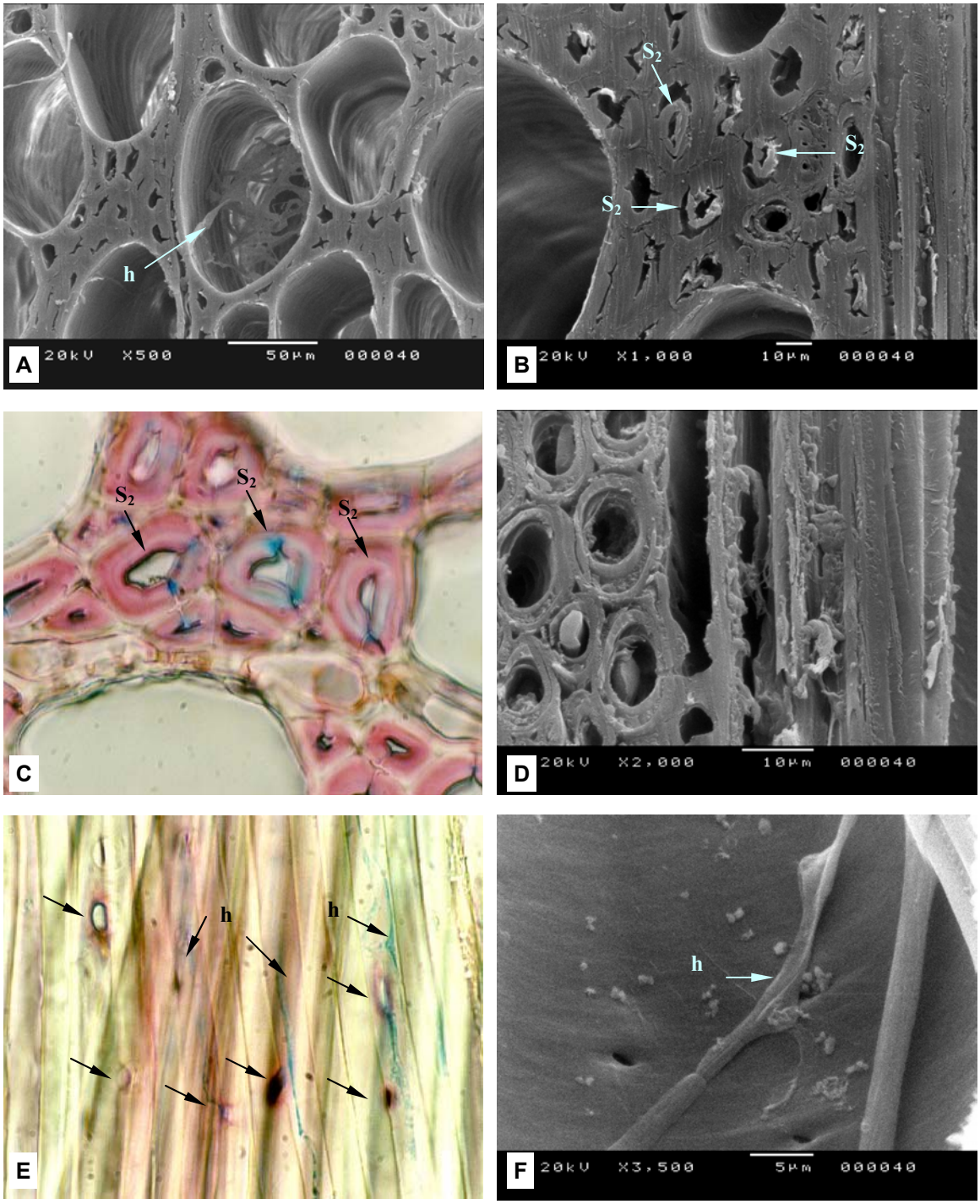
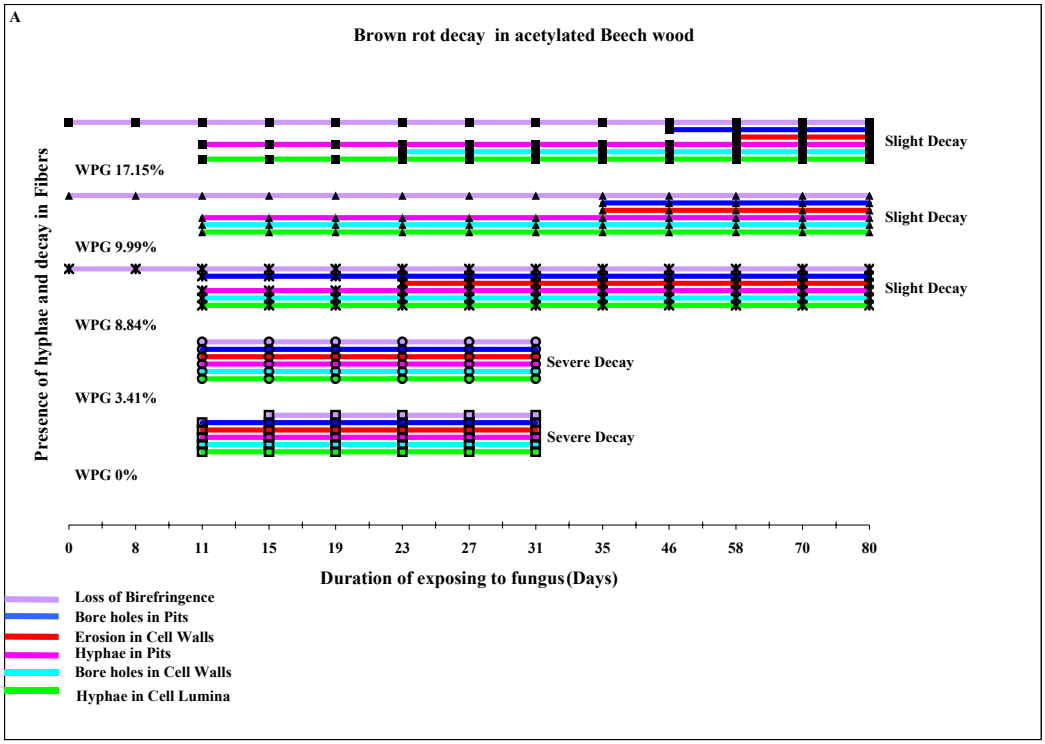


Fig. 4-17. Brown rot decay in acetylated beech; WPG 17.15%. **A-C:** Early stage of brown rot decay in highly acetylated beech wood. **A:** Hyphae (h) colonize into vessel lumina. **B:** S_2 layers are modifying due to enzymatic activities. Modified S_2 layers have been detached from cell wall due to SEM preparation procedure. **C:** S_2 layer in fiber cells are modifying due to enzymatic activities. Changing the red color in secondary walls into bluish color reveals a rapid removal of lignin and slow decay in polysaccharides (arrows). **D:** Ray cells are still intact. **E&F:** Hyphae (h) could produce bore holes (arrows) on the cell walls. **A&B:** Cross view; SEM. **C:** Cross section. **D:** Tangential view; SEM. **E:** Longitudinal view. **F:** Radial view; SEM.



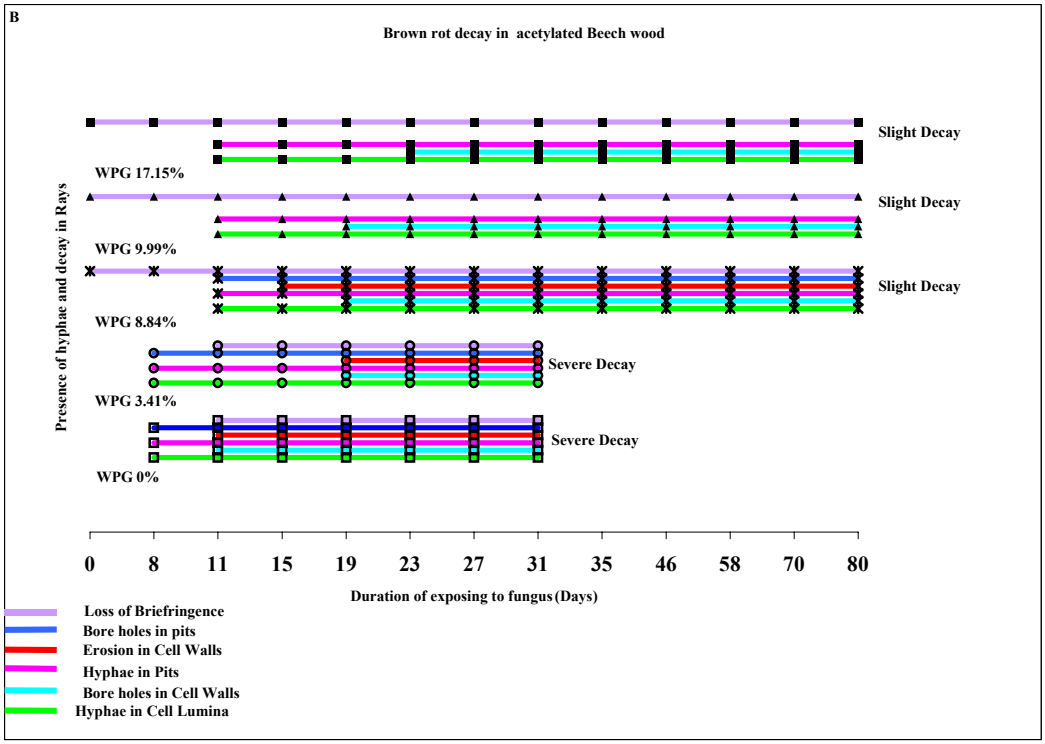


Fig. 4-18. Advancement of brown rot decay (*Poria placenta)* in acetylated beech wood; **A**: Fibers; **B**: Rays

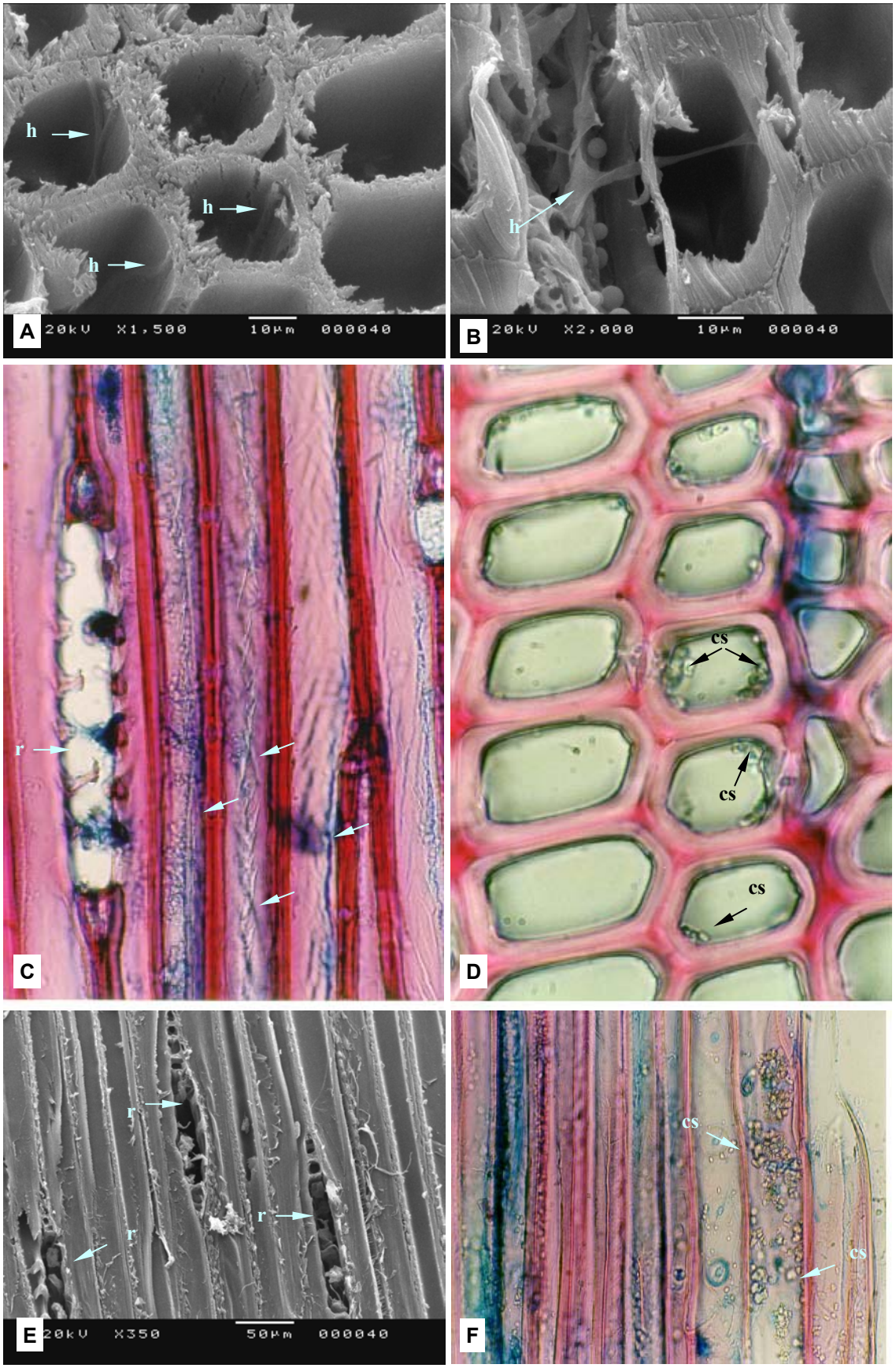

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Fig. 4-19. Severe brown rot decay in non-acetylated Scots pine. **A:** Due to extensive degradation produced by hyphae (h), cell walls have porous structure. **B:** Hyphae (h) penetrate into cells through rays and cross-field pits. **C&E:** Rays (r) have been severely decayed and removed. **C:** Cracks on tracheid cell walls show severe attack by fungus (arrow heads). **D&F:** Hyphae (h) produce oxalate crystals (cs) to support chemical degradation during decay. **A&B:** Cross view; SEM. **C:** Tangential section. **D:** Cross section. **E:** Tangential view; SEM. **F:** Radial section.

Table 4-3 represents advancement of brown rot decay in the acetylated beech wood. Advanced brown rot decay was recognized in the non-acetylated wood and the lowest weight gain. In both cases, wood severely decayed. The attack was so aggressive that majority of cells were lost their integrities after 31 days (fig. 4-18A&B). Therefore acetylation at weight gain 3.41% was categorized with "no protection". The level of acetylation for its protection in moderately acetylated wood was placed at "good protection" due to slight decay in fiber cell walls and appeared bore holes in fibers and rays. Because of initial stage of brown rot decay at the highest weight gain (17.15%) and observed modifying cell walls and formed bore holes, the acetylation was categorized in "better protection" level.

Scots pine- Figure 4-19 represents severe brown rot decay in non-acetylated pine wood. Tracheids were extensively decayed by brown rot hyphae, which were located in cell lumina. Due to heavy attack and removal of polysaccharides, cell walls in tracheids showed porous structures (fig. 4-19 A). Hyphae colonized into rays and penetrated to other cells by using cross-field pits (fig. 4-19 B). Severe decay was occurred in ray cells and empty rays were remained after attack (fig. 4-19 C&E). Many oxalate crystals were observed in tracheid lumina and around hyphae (fig. 4-19 D&F). Production of oxalates supports chemical degradation of wood by brown rot fungus *Poria placenta*. Many cracks were appeared on tracheid walls (fig. 4-19 C&F). This occurrence also indicates severe brown rot decay in the non-acetylated Scots pine wood.

Extensive brown rot decay was also observed at the lowest degree of the acetylation (WPG 2.71%) (fig. 4-20). Broken and collapsed cells were observed (fig. 4-20 A). Cell walls had porous structures after extensive attack. It indicates removal of polysaccharides. However using mixture of safranin-astra blue stain revealed initial removal of lignin by changing the red color to blue (fig. 4-20 B). Blue color in S_2 layer indicates the presence of polysaccharides. S_3 layers were remaining intact before advanced stage of decay (fig. 4-20 B), while they were removed in advanced stage of decay (fig. 4-20 A). Hyphae could produce bore holes to penetrate into tracheids (fig. 4-20 C&D). Severe attack was also observed in ray cells (fig. 4-20 E). After attack the rays were remained empty.

In moderately acetylated wood (WPG 9.97%), brown rot decay was also observed (fig. 4-21). However it was not as severe as the lowest WPG (2.71%). Main decay occurred in tracheids that were surrounding resin canals (fig. 4-21 A). Modification of S_2 layer was also distinguished in tracheids (fig. 4-21 B). In some cells, the color of S_2 layer was changing into blue. As it was explained, this indicates that delignification process occurs in cell walls (fig. 4-21 B) by the brown rot fungus. Bore holes were also observed in tracheid walls (fig. 4-21 C). Ray cells were attacked and removed by brown rot fungus (fig. 4-21 D). Their empty places were remained after attack. It was observed that hyphae erode pit membranes (fig. 4-21 E). Oxalate crystals, which were surrounding the hyphae, were also observed in cell lumina (fig. 4-21 F). Oxalate production supports chemical modification of the cell walls. At WPG 10.97%, brown rot attack was also distinguished (fig. 4-22). In this case, attack was also scarce and broken cells were close to resin canals (fig. 4-22 A). Figure 4-22 B indicates modification of S_2 layers. S_3 layers were seen intact (fig. 4-22 B). It was also observed that hyphae produce bore holes to pass the cell walls (fig. 4-22 C&D). Cracks and erosions were also distinguished in cell walls (fig. 4-22 C&D). However it was not as severe as the non-acetylated wood and the lowest WPG. Ray cells were seen attacked and their empty places were also sometimes distinguished (fig. 4-22 E).

AT the highest WPG (19.65%), no brown rot decay was distinguished in tracheid cell walls (fig. 4-23 A). While, microscopy showed that hyphae could colonize into cell lumina (fig. 4-23 A, B&F) and produce oxalate crystals (fig. 4-23 B&E). Oxalate crystals were seen in different shapes, such as rhomboid, needles,

clusters, etc (fig. 4-23 E). Rays were attacked by brown rot hyphae and some times their empty places were remained after decay (fig. 4-23 C&D). Bore holes were seen in cell walls (fig. 4-23 G&H). Our observations represent initial stage of brown rot attack in the highly acetylated Scots pine wood.

Study on the advancement of brown rot decay in acetylated Scots pine showed that colonization of hyphae occurred rapidly after 8 days of incubation in tracheids of non-acetylated and acetylated wood (fig. 4-24A). Erosion of cell walls began after 11 days in the non-acetylated wood, while it was after 15 days at WPG 2.71% and 19 days later at WPG 9.97%. Tracheids began to be eroded after 58 days of incubation in moderately acetylated wood (WPG 10.97%), while no erosion was observed in the highly acetylated tracheids (WPG 19.65%). Formation of the bore holes occurred within the first week of the colonization when hyphae were placed in cell lumina and pit chambers (fig. 4-24A) of acetylated and non-acetylated pine wood. Number of bore holes were enormous in non-acetylated wood comparing the acetylated ones. Study of brown rot attacked wood under polarized light showed that loss of birefringence occurred after 15 days of incubation in the non-acetylated and the low acetylated tracheids (WPG 2.71%). Similar to samples of the white rot trial, intact and unexposed samples of pine showed that acetylated tracheids lose their birefringence properties at weight gains above 9.97%. This evidence also reveals disadvantage of polarized microscopy in detection of decay at early stages of brown rot. The observation will be discussed in details.

Colonization of brown rot hyphae were recognized within the first week of the incubation in rays of the acetylated and the non-acetylated pine wood (fig. 4-24B). Ray cells in the non-acetylated wood were eroded after 11 days and in the acetylated wood, it was after 15 and 19 days at WPGs 2.71% and 9.97% respectively. Erosion of ray cells occurred after 31 and 35 days in moderately (WPG 10.97%) and highly acetylated wood (WPG 19.65%) respectively. Brown rot fungus could invade rays in the non-acetylated wood within 19 days and the invasion of the rays was within 23 days of the incubation at weight gains 2.71% and 9.97% respectively. And after 58 days of the incubation, rays were removed by fungus at WPGs 10.97% and 19.65%. Severe attack of rays indicates aggressiveness of the brown rot fungus *Poria placenta* in attacking the non-acetylated and the acetylated pine wood.

Study on the phenology of brown rot decay in acetylated pine wood (table 4-3) placed the lowest degree of the acetylation in “no protection” level due to severe brown rot attack. Occurrence of slight erosion of tracheid cell walls and less number of bore holes placed moderate acetylation (weight gains 9.97% and 10.97%) in “good protection” level. While the acetylation at the highest weight gain was placed in “well protection” due to no erosion of tracheids, few numbers of bore holes and disappearing ray cells. This investigation showed that brown rot fungus can easily colonize in tracheid lumina and ray cells of the acetylated pine wood.

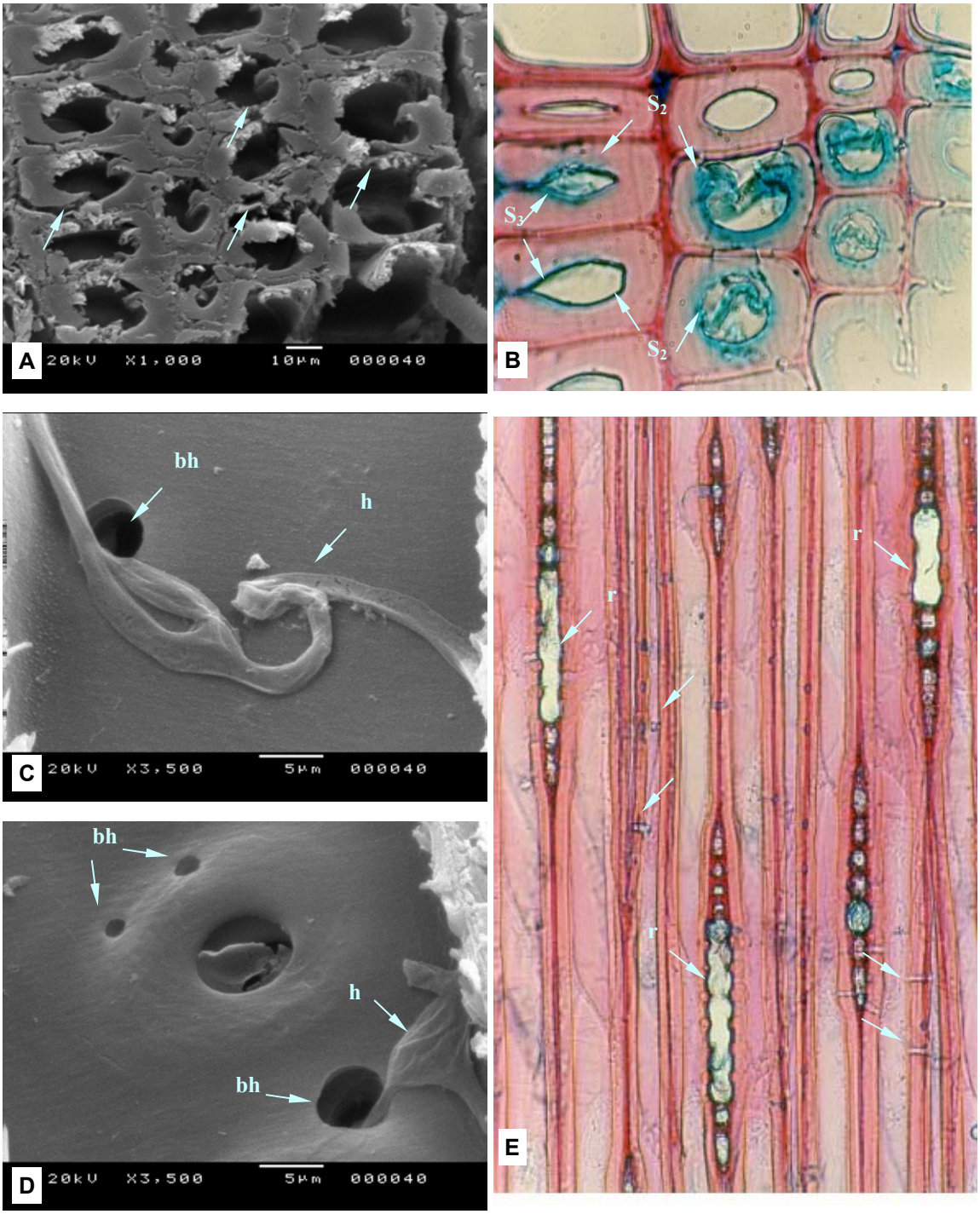


Fig. 4-20. Severe brown rot decay in acetylated pine; WPG 2.71%. **A:** Broken and collapsed cell walls and their porous appearance indicate a heavy attack on cell walls (arrows). **B:** S_2 layers are being modified due to enzymatic activities, while S_3 layer seems intact. Blue color in S_2 layers indicates a delignification process. **C&D:** Hyphae (h) produce bore holes (bh) to penetrate into the cell walls and degrade them. **E:** Brow rot fungus degrades ray cells (r) and leaves empty rays after a heavy attack. **A:** Cross view; SEM. **B:** Cross section. **C&D:** Radial view; SEM. **E:** Tangential section.

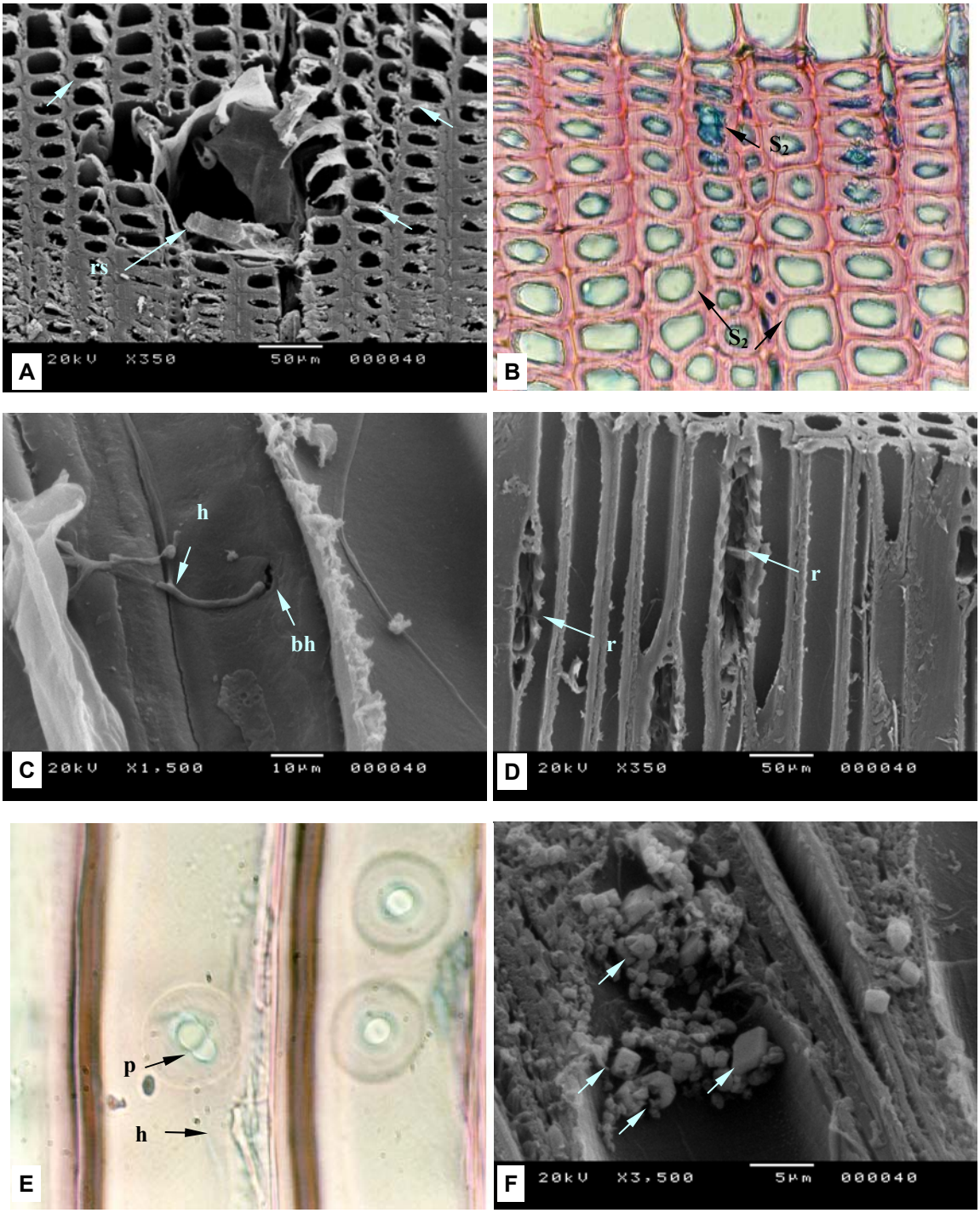


Fig. 4-21. Brown rot decay in acetylated pine; WPG 9.97%. A: Close cells to resin canal (rs) have been degraded severely. Collapsing and broken cells (arrows) with porous structures reveals developing stage of brown rot decay in tracheids. B: Reducing red color in S_2 layer of some cells indicates removal of lignin and polysaccharides. Blue color in S_2 layer of other cells reveals that delignification has been occurred in cell walls. C: Hyphae (h) produce bore holes (bh) on cell walls. D: After heavy attack of ray cells by brown rot fungus, rays (r) remain empty. E: Hypha (h) erodes pit membrane (p). F: Oxalate crystals (arrows) in tracheid lumen. A: Cross view; SEM. B: Cross section. C: Radial view, SEM. D: Tangential view; SEM. E: Radial section. F: Radial view; SEM.

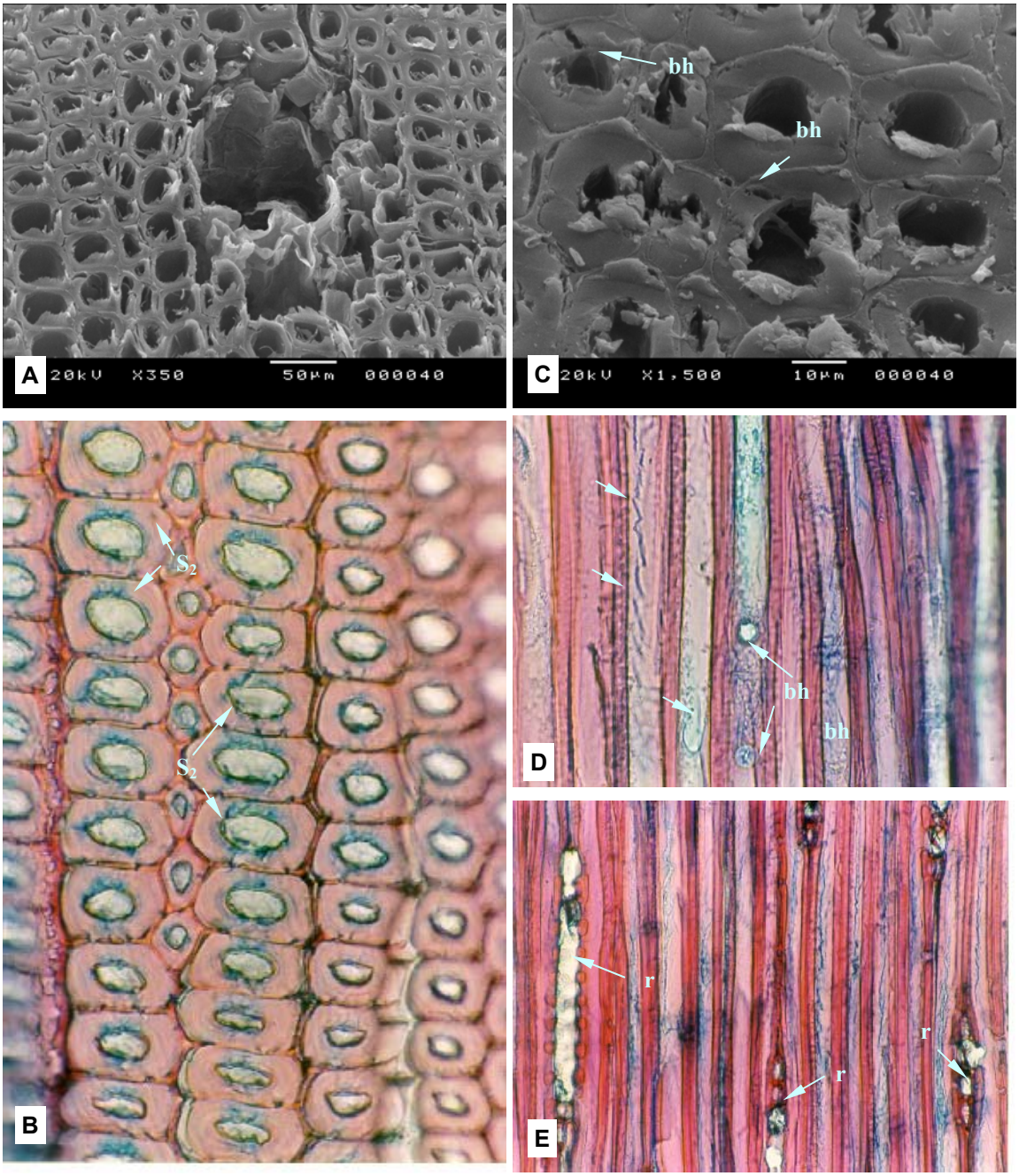
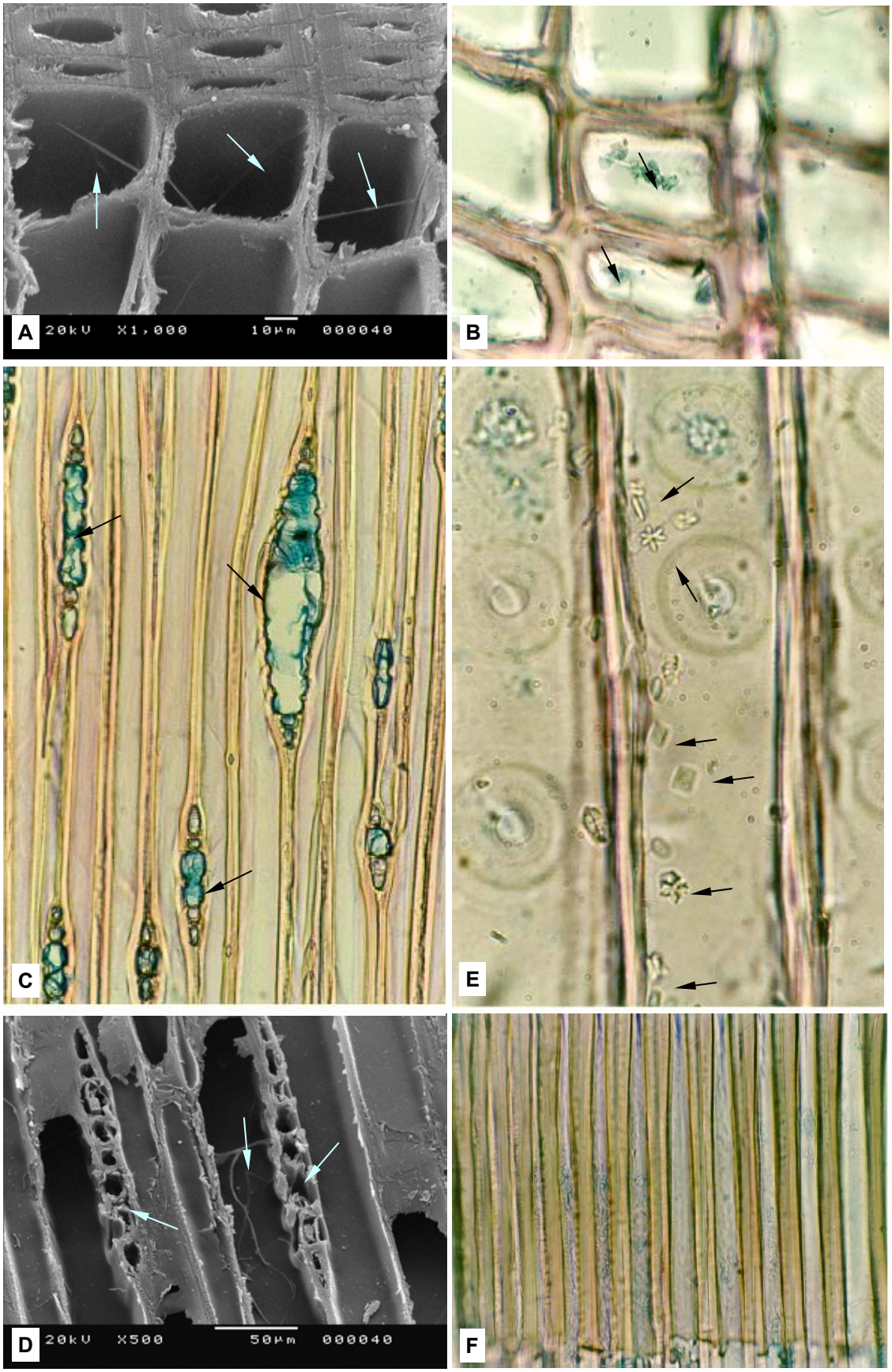


Fig. 4-22. Brown rot decay in acetylated pine; WPG 10.97%. **A&C:** Collapsing and broken cell walls indicate brown rot decay in cell walls. Hyphae produce bore holes (bh) in tracheid walls. **B:** Modifying S_2 layers with blue color indicates removal of lignin during brown rot decay. **D:** Cracks, erosion (arrows) and bore holes (bh) on tracheids represent heavy attack in cell walls. **E:** After a severe brown rot decay rays (r) remained empty. **A&C:** Cross view; SEM. **B:** Cross section. **D:** Radial section. **E:** Tangential section.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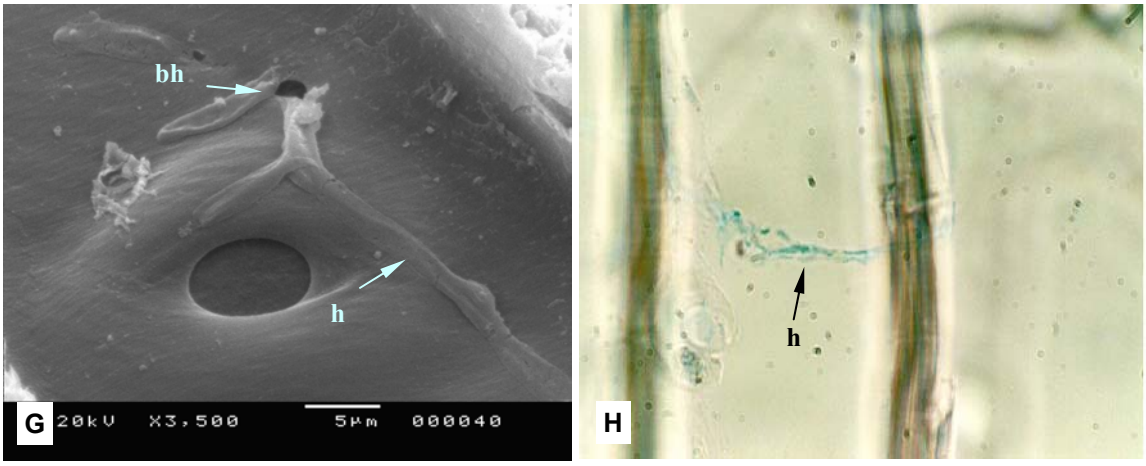

Fig. 4-23. Brown rot decay in highly acetylated pine; WPG 19.65%. **A&B:** Hyphae (h) could colonize into vessel lumina and produce oxalate crystals (arrows). However there is no decay in cell walls. **C&D:** Hyphae degrade rays at early stage of decay. **E:** Oxalate crystals have been produced in different shapes, needle form, clusters, rhomboid. **F:** Hyphae colonize into tracheid lumina. There is no cell walls erosion. **G:** Production of bore holes (bh) by hyphae on tracheids represents early stage of brown rot decay in highly acetylated pine. **H:** Hyphae cross tracheids by bore hole production. **A:** Cross view; SEM. **B:** Crosse section. **C:** Tangential section. **D:** Tangential view; SEM. **E&F:** Radial section. **G:** Radial view: SEM. **H:** Radial section.

4.4. Discussion

Concerning the microscopical observations, it can be concluded that hyphae of both fungi can easily colonize in cell lumina and ray cells. They use open ways to penetrate into wood, preferentially vessel lumina and rays at earlier stage and then penetrate into fiber cell lumina through inter-fiber pits or cross-fields between rays and fibers. Rays in the non-acetylated beech wood and probably at low weight gains have enough nutrients for hyphal growth. However in moderately and highly acetylated wood, those nutrients were probably removed during the acetylation process and no or insufficient nutrients were remained for fungal growth in ray cells. Lack of the nutrients and protection of the ray cell walls against fungi due to the acetylation could be the main reasons for slight or no decay in the ray tissues of the moderately and highly acetylated wood.

Any change in the cell wall birefringence indicates that crystalline cellulose has been attacked (Anagnost, 1998 & Wilcox, 1993a&b, Wilcox, 1968). In the non-acetylated wood and at the lowest weight gain, the loss of birefringence occurs in cell walls due to fungal attack at early stage of decay. However concerning figures 4-6, 4-12, 4-18 and 4-24 (A&B), wood cell walls have lost their birefringence before any attack at weight gains above 8.84% in beech and 9.97% in pine wood. This indicates that crystalline cellulose was influenced due to the acetylation process. Probably the occurring substitutions of hydroxyl groups in crystalline cellulose with acetyl groups due to the acetylation have changed its stereo-chemical structure, which could influence the polarization behavior of crystalline cellulose under polarized light and sequentially the birefringence property of acetylated cell walls was changed due to the acetylation. The birefringence property of the cell walls at the lowest WPGs is very similar to the non-acetylated wood. This reveals that the substitutions were not so effective to change the stereo-chemical structure of crystalline cellulose at low weight gains. Kumar & Agarwal (1983) reported that at weight gain of 13.5% in acetylated wood, about 86.4% of hydroxyl groups in lignin, 21.6% in hemicelluloses and 9.3% in cellulose are substituted. Polarized microscopy here confirms the former report in which crystalline cellulose was influenced due to the substitution of acetyl groups. Our observations in the acetylated wood reveal the disadvantage of using this property solely as an indicator for identification of any decay patterns under polarized light in acetylated wood.

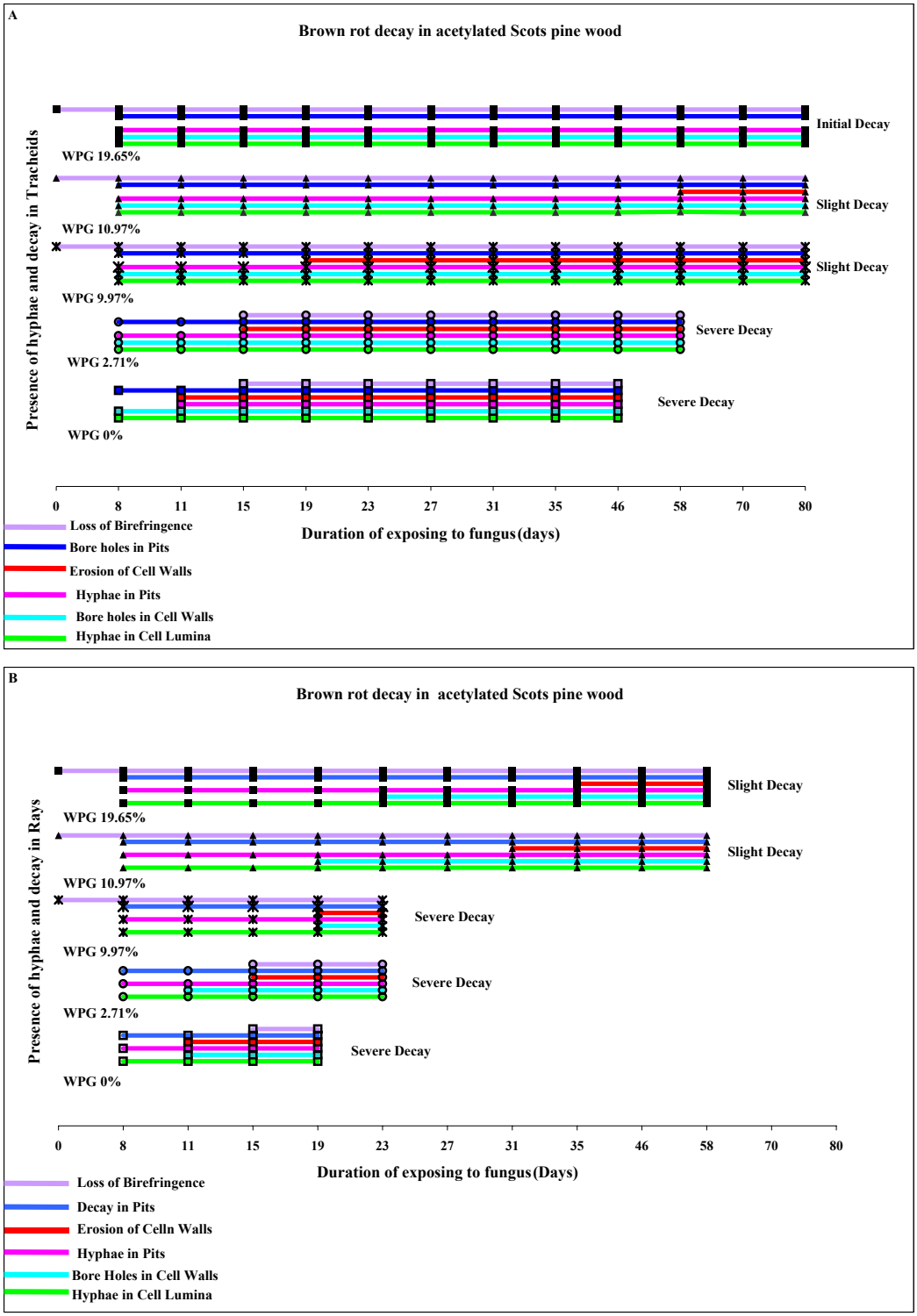

Fig. 4-24. Advancement of brown rot decay (*Poria placenta)* in acetylated Scots pine wood; **A**: Fibers; **B**: Rays

Table 4-2. Advancement of white rot decay in acetylated wood

Species	WPG %	Stage of white rot decay *Trametes versicolor*					Stage of decay	Acetylation protection
		Colonization of hyphae in cell lumina	Colonization of hyphae in rays	Cell wall erosion	Opened pits	Decayed rays		
Beech	0	+	+	+	+	+	Severe	-
	3.41	+	+	+	+	+	Severe	No protection
	8.84	+	+	Scarce	+	+	Slight	Good protection
	9.99	+	+	Slight	-	+	Slight	Better protection
	17.15	+	+	-	-	-	No decay	Best protection
Scots pine	0	+	+	+	+	+	Severe	-
	2.71	+	+	+	+	+	Severe	No protection
	9.97	+	+	Slight	+	+	Slight	Good protection
	10.97	+	+	Scarce	- / Small bore holes in tori	+	Slight	Better protection
	19.65	+	+	-	-	Scarce	Initial stage of decay	Well protection

Table 4-3. Advancement of brown rot decay in acetylated wood

Species	WPG %	Stage of brown rot decay Poria placenta					Stage of decay	Acetylation protection
		Colonization of hyphae in cell lumina	Colonization of hyphae in rays	Cell wall erosion	Bore holes	Decayed rays		
Beech	0	+	+	+	+	+	Severe	-
	3.41	+	+	+	+	+	Severe	No protection
	8.84	+	+	Slight	+	-	Slight	Good protection
	9.99	+	+	Slight	+	-	Slight	Good protection
	17.15	+	+	Modifying cell walls	+	-	Initial stage of decay	Better protection
Scots pine	0	+	+	+	+	+	Severe	-
	2.71	+	+	+	+	+	Severe	No protection
	9.97	+	+	Slight	+	+	Slight	Good protection
	10.97	+	+	Slight	+	+	Slight	Good protection
	19.65	+	+	-	+	+	Initial stage of decay	Well protection

Concerning the figures (4-18 & 4-24), rapid invasion of tracheids and rays in the non-acetylated pine wood earlier than the beech wood indicates that the brown rot fungus has a preferential tendency to attack pine (as a softwood) than that the beech wood (as a hardwood). Whereas, the preference of the white rot fungus is to degrade the beech wood than that of the pine wood. Microscopy also showed that each fungus had its own preference. The brown rot fungus attacked non-acetylated pine wood easier than beech wood. For example vessels and rays in beech were not severely attacked as tracheids and rays in pine wood (figs. 4-13 & 4-19 and tables 4-2 & 4-3). The similar preference was followed in the acetylated wood. Whereas the white rot fungus attacked beech wood easier than pine wood. Because fiber cell walls were severely eroded and removed by *T. versicolor* in beech wood, while tracheids could resist their integrities after severe attack (figs. 4-1 & 4-7 and tables 4-2 & 4-3). Similar preferential attack by white rot fungus was also distinguished in acetylated beech and pine. Same preferences in attacking acetylated soft and hardwoods were reported by some authors (Okino *et al.*, 1998; Suttie *et al.*, 1998). Schwarze *et al.* (2000) and Green & Highley (1997) reported that brown rot fungi attack softwoods more preferential than hardwoods and white rot fungi have more interest to attack hardwoods than softwoods. Ohkoshi *et al.* (1999), Takahashi *et al.*, (1989a&b), Takahashi (1996) and Okino *et al.* (1998) have also reported that brown rot fungi degrade acetylated softwood faster than hardwoods and white rot fungi are more resistant to acetylated hardwoods than softwoods. A possible involvement of different chemical reactivities between the two types of lignin, namely the guaiacyl (softwood lignin) and the syringyl (hardwood lignin), and/or possible different cellular distributions of acetyl bond between softwood and hardwood are suggested. Investigations have shown that syringyl lignin was degraded more rapidly than that guaiacyl lignin (Zabel & Morrell, 1992).

Microscopical studies of white- and brown-rot decayed wood showed that the acetylation at low weight gains (3.41% and 2.71% in beech and pine respectively) gives no protection to acetylated wood against both fungi and they could attack both wood species as severely as the non-acetylated one. Using the moderate acetylation process to achieve weight gains under about 10% in both species gives considerable protection to wood when it is compared to the low acetylation levels. Acetylation of wood at about above 10% of weight gains demonstrated interesting reduction and inhibition in wood decay. In order to reaching to prevent decay, acetylation levels higher than the achieved weight gains here are required.

Reports have shown that the acetylation improves the bioresistance of wood against soft rot fungi in pine, poplar and beech wood at weight gains above 10.7%, 14.4% and 12.8% respectively (Beckers *et al.*, 1995). Similar results have also been reported for beech and pine wood against soft rot fungi. Weight gains above 8% for beech and 10% for pine had considerable influences against soft rot fungi in soil test (Mohebby & Militz, 2002). Ohkoshi *et al.* (1999) showed that the decay by brown rot fungus (*Tyromyces palustris*) became inhibited at a weight percent gain higher than 10% and the mass loss due to decay became zero at a WPG more than 20%, while the weight loss due to decay to white rot fungus (*Coriolus versicolor*) decreased slowly with the increase in WPG and reached zero at about 12%. Militz (1991) also studied the effect of the acetylation at weight gain 20.8% in beech wood and reported that brown rot fungi (*Gloeophyllum trabeum*, *Poria placenta* and *Coniophora puteana*) failed to attack the acetylated wood. Takahshi *et al.* (1989) tested weight loss in acetylated wood and showed that acetylation between weight gains 10-15% had striking decrease effect on brown rot fungus *Tyromyces palustris* and prevented decay at WPG 20%, while the striking decrease level for *Coriolus versicolor* was between 12-15% and prevention at WPG 20% in non-durable wood species. Another report showed that the protection level was above 6% in acetylated wood (Kumar, 1994). Nilsson *et al.* (1988) also reported a weight gain of 15% could reduce white and brown rot fungal attacks in acetylated particleboards. Resistance of sweetgum was tested by Kalnins (1982) in soil for 12 weeks using two brown rot fungi (*Gloeophyllum trabeum* and *Lentinus lepideus*) and a white rot fungus (*Coriolus versicolor*). Tests showed a very good resistance against those fungi when the weight gain reached above 15%. Okino *et al.* (1998) showed that acetylated pine and eucalypt flakeboards at weight gains 16-18% prevented against white- and brown-rot fungi. Several workers have reported that an acetylation level of 17% weight gain is adequate to control fungal decay and 10% was the efficient level to achieve a good protection (Goldstein *et al.*, 1961; Peterson & Thomas, 1978).

Earlier, authors believed that bioresistance mechanism of the acetylated wood is related to changing the hydrophilic nature of wood and reducing moisture contents in cell walls due to substitution of hydroxyl groups with acetyl groups (Stamm & Baechler, 1960; Rowell, 1983). Foster *et al.* (1997) and Foster (1998) tested different chain anhydrides and reported that the bioresistance of acetylated wood is related to

"bulking effect" of the chemical reagents during wood modification. Bulking of cell walls blocks small pores or reduces their sizes to smaller than the required sizes for enzyme penetration or blocking of cell wall micro-capillaries prevents the access of low molecular weight degradative agents produced by the fungus (Papadopoulos & Hill, 2002; Hill, 2002). Another hypothesis has related the protection mechanism of the acetylation to "chemical alteration" in cell wall polymers with blocking the active sites and making them inaccessible to fungal enzymes (Takahashi *et al.*, 1989a&b; Takahashi, 1996).

Regarding the visualized observations, brown- and white-rotten acetylated samples were seen wet enough to provide the optimal moisture for fungal decay condition. Or at least S_3 layer in pine tracheids and vessels in beech or luminal sides of other cells, which were in direct contact with hyphae, had enough moisture to be attacked by fungi. Concerning the pattern of white rot decay in the non-acetylated wood, the white rot fungus thinned cell walls from lumina inward the ML. Therefore, luminal sides of the acetylated cells were wet enough to be attacked by the fungus. Whereas neither tracheid cells were not severely attacked, nor vessels were remained protected at the highest weight gains. The origin of the moisture in samples is due to the breakdown of cell wall polymers in attacked or adjacent samples (Suttie *et al.*, 1997&1998). Therefore, this hypothesis could not be confirmed alone. On the other hands, slight brown rot attack and bore holes on the highly acetylated cell walls do not entirely confirm the hypothesis of "bulking effect" or "blocking of micro-capillaries" and reduction of small pores in the cell. Brown rot fungi use non-enzymatic method by producing free radicals from Fenton reaction which are smaller than the smallest pore sizes (< 3.8 nm) in wood cell walls (Flournoy *et al*, 1991; Highley *et al.*, 1994) and can easily penetrate into cell walls. Because, the smallest enzymes of brown rot fungi are too large to penetrate through the cell wall pores (Highley *et al.*, 1994). An average size for conventional cellulases is about 5nm in diameter if the molecule is assumed to be spherical in shape or 3 by 17nm if ellipsoid. The size for lignin peroxidases is 4.7nm if their shape is considered to be spherical and 4.3 by 6nm if the shape is considered to be ellipsoid. Manganese peroxidases have similar sizes as lignin peroxidases. Therefore, the evidences indicate that those enzymes cannot penetrate into cell wall pores, which are with 2nm sizes (Flournoy *et al.*, 1993). However, small cellulases (1.5 to 4nm) and ellipsoid cellulases may thus gain access to cell walls at early stages if white rot decays wood and small molecule radical can diffuse into cell walls if brown rot fungus decays wood (Flournoy *et al.*, 1993). It was reported that oxalates support non-enzymatic decay system in wood cell walls at initial stage of decay by supporting the chemical reactions which provide some free radicals (Green *et al.*, 1998; Koenig, 1974; Highley *et al.*, 1994). Production of oxalate crystals in highly acetylated tracheid cell lumina indicates non-enzymatic activity of brown rot fungus. Brown rot fungi have the capability to produce extracellular oxidative metabolites when the organisms are maintained under specific conditions, especially nitrogen and carbohydrate limitations (Highley *et al.*, 1994).

Consequently, the capability of the brown rot fungus to attack acetylated wood at higher weight gains in comparison with white rot fungus could be related to its non-enzymatic system. This fungus can attack acetylated wood by producing oxalates and free radicals, which are smaller than enzymes. Concerning the mode of action in white rot decay, the presence of hydroxyl groups in cell walls is very important in initializing the attack by hydrolyzing enzymes (Eriksson *et al.*, 1990; Highley & Dashek, 1998). More acetyl substitutions in lignin, in comparison with cellulose, probably limit its ligninolytic activities, whereas brown rot fungus has more cellulolytic capability than ligninolytic (Eriksson *et al.*, 1990; Srebotnik & Messner, 1991). Therefore, less acetyl substitutions in carbohydrates (especially cellulose) permit brown rot fungus to attack acetylated wood even at higher weights gains. Rosenqvist (2001) proved by using labeled ^{14}C and tritium acetic anhydride that uniform substitutions were seen under scanning electron microscope at higher weight gains (above 20%). Also Kumar & Agarwal (1983) reported uneven substitutions in cell walls components during the acetylation. Their report showed that acetylation of lignin is faster than hemicellulose and holocellulose (lignin>hemicelluloses>holocellulose) during the acetylation and at same weight gains substitution of hydroxyl groups in lignin is more than hemicellulose and cellulose (e.g. at 13.5% of weight gain, 86.4% of hydroxyl groups in lignin, 21.6% in hemicelluloses and 9.3% in cellulose are substituted. In another report, Rowell *et al.* (1994) showed that at 18% of weight gain, almost all lignin hydroxyl groups were substituted, but only about 20% of the holocellulose hydroxyls were substituted). Therefore Kumar & Agarwal (1983) suggested that higher lignin substitution indicates that acetylated wood should be more resistant to white rot fungi than brown rot.

Due to the decreased number of hydroxyl groups and smaller pore sizes at higher weight gains, cellulolytic and ligninolytic enzymes of white rot fungus can probably not diffuse into cell walls and fail to

attack at higher weight gains. However the observed slight brown rot decays at higher weight gains and the scarcely produced bore holes on the highly acetylated cell walls puts theories of bulking effects and blocking of micro-capillaries under question. Therefore, it is probably suggested that whole cell walls were not uniformly acetylated during the acetylation. Probably, there exist some small regions in the cell walls were not acetylated as high as others and fungi could search for such small regions with low levels of acetylation to attack (concerning the produced bore holes by the brown rot fungus). Finally we could suggest complexity of factors that involve in protecting the acetylated wood against fungi such as lowered moisture content in cell walls that influences hydrolyzing enzymes, blocking of active sites in cell wall polymers that can influence some radical reactions, bulking effect and blocking of micro-capillaries (micro-pores) in cell walls that inhibit diffusion of small degrading agents and small regions in cell walls that gained lower degrees of acetylation in comparison with other close regions.

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Chapter 5
Bioassays of acetylated wood

5.1. Introduction

Hydroxyl groups have been believed as reactive sites in wood polymers, which are responsible for absorption of surrounding humidity that provides dimensional instability. And also they are known as enzyme reactive sites (Takahashi, 1996). Due to the acetylation, those hydroxyl groups were substituted by hydrophobic acetyl groups and wood becomes protected against absorbing surrounding humidity and decay by microorganisms, e.g. fungi, etc. It is expected that fungi do not degrade wood due to this protection and due to lowering the moisture content in wood below the fungal limiting moisture contents. By now, many reports showed and supported bioresistant property of acetylated wood. However, it depends on the degree of acetylation. Reports have indicated that increasing degree of the acetylation in wood, which is indicated by the WPG (weight percent gain), reduces or inhibits fungal activities. Fungal testes in different acetylated wood species have shown that fungi could not decay acetylated wood at higher weight gains. However, they can colonize in those woods (Mohebby & Militz, 2002). The mode of fungal action in acetylated wood is still unknown and it is not clear whether fungi could live in highly acetylated wood and how they decay it.

Therefore, this research was conducted to monitor fungal activity in acetylated wood by using different sensitive methods, such as enzyme assays, protein assays, microcalorimetry. White rot fungi produce different ligninolytic enzymes. Laccase and manganese peroxidases (MnP) are major involve in lignin decay. Cellulases, xylanase and mannase are responsible for polysaccharide degradation. *Trametes versicolor* as a standard fungus produces laccase (benzenediol:oxygen oxidoreductase, Enzyme Commission (EC) Number: 1.10.3.2), LiP (lignin peroxidases, ligninase, EC 1.11.1.7), MnP (manganese peroxidases, EC 1.11.14), cellulases, xylanase and mannase (Hatakka, 1994; de Koker *et al.*, 2000). In this study, the white rot fungus *Trametes versicolor* was used to investigate its bioactivity. The activity of enzymes, which are responsible for wood decay, was studied in order to see to which extent they are produced in the acetylated wood.

5.2. Material and methods

5.2.1. Sample preparation

Mini-stakes from beech (*Fagus sylvatica*) sapwood (5×5×110mm) were prepared and dried at 60°C to get homogenous moisture content before acetylation and then they were acetylated according to the method that was explained in section 2.2. The obtained weight percent gains were 0.0, 2.0, 9.0, 10.2 and 17.0%.

Main samples were prepared as follow:

- *Solid-state fermentation*: Mini-blocks were cut from the mentioned acetylated mini-stakes (5×5×20mm) and their initial dry weights were measured before sterilization.
- *Liquid-state fermentation:* Rest of cut mini-stakes were milled by a Retsch ZM100 mill to pass mesh 40. About 5g of each weight gain was weighed and packed with 6 replicas before sterilization.

5.2.2. Sterilization

The prepared mini-blocks and packed milled wood were sterilized by autoclaving at 121°C for 20 minutes.

Two series of experiments (solid- and liquid-state fermentation) were performed in this study as follows:

5.2.3. Solid-state fermentation

5.2.3.1. Medium and fungus

Glucose malt extract (ME) plates (per liter: 15.0g agar, 10.0g glucose, 3.5g malt extract) were prepared and autoclaved at 121°C for 20 minutes. The ME plates were incubated at 23°C and 70% relative humidity. Agar plugs (5mm diameter) were punched from the leading edge of the mycelium grown on ME plates and used as inoculums for the experiments (Dorado *et al.*, 2000).

Twenty sterilized test mini-blocks were introduced to the plates carefully for each weight gain (fig. 5-1A) and incubated at 23°C and 70% relative humidity for 12 weeks period. Sampling was carried out in 2 weekly intervals for following studies.

Fig. 5-1. Fungal plate for soild-stae fermentation test (A) and Liquid culture flasks for liquid-state fermemntation test (B)

5.2.3.2. Mass loss determination

At every sampling term, 10 mini-blocks were used for mass loss determination. Samples were dried carefully in a vacuum chamber at about 50°C to avoid any effect on ergosterol content. After weight loss determination samples were used for ergosterol assay. Mass losses were calculated based on initial weights of the samples.

5.2.3.3. Ergosterol assay

Ergosterol is a sensitive method to determine fungal biomass in wood. After mass loss determination, samples were cut into small particles by a sharp knife and homogenized by an Ultra-Thurrax in liquid nitrogen. For ergosterol assay, the same method was used which has been explained in section 2.3.7. Ergosterol content was calculated based on wood dried weight.

5.2.3.4. Microcalorimetry

Three mini-blocks were separately incubated at the same conditions as above to measure fungal heat production in acetylated wood. The same method was used for microcalorimetry, which has been explained in the sections 2.3.6.

5.2.3.5. Fluorescein diacetate analysis (FDA)

Fluorescein diacetate (FDA, 3,6-diacetoxyfluoran, $C_{24}H_{16}O_7$) has been used to determine esterases activity in living microbial cells. FDA is hydrolyzed by a number of different enzymes, such as proteases, lipases and esterases. The product of this enzymatic conversion is fluorescein ($C_{20}H_{12}O_5$). Fluorescein can be quantified by fluorometry or spectrophotometry (Schnürer & Rosswall, 1982; Kerem *et al*, 1992; Swisher & Carroll, 1980). FDA has been used in this study as another sensitive tool to quantify fungal activity and monitor their colonization in acetylated wood.

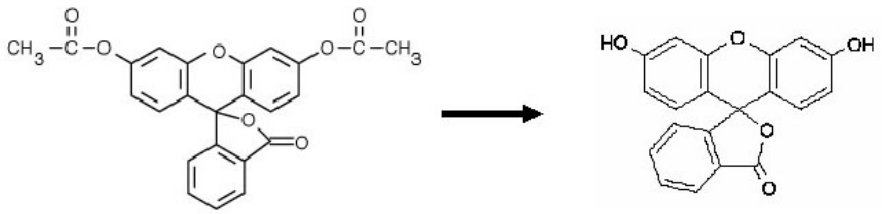

Fig. 5-2- Fluorescein diacetate (FDA) or 3,6-diacetoxyfluoran ($C_{24}H_{16}O_7$); left and fluorescein ($C_{20}H_{12}O_5$); right

At each sampling term, ten mini-blocks were used for FDA analysis. They were homogenized by an Ultra-Thurrax. Some samples were used to determine the wood moisture content to calculate wood oven dry weight. FDA (Sigma Chemical Co., Catalogue no. F7378) was dissolved in acetone (analytical grade) to reach 2mg/ml concentration of solution and stored at -20°C as a stock solution in different small aliquots for each usage. Triplicates of homogenized samples (1g) were dispersed in 100ml of sterile 60mM sodium phosphate buffer solution (pH 7.6) in a 250ml Erlenmeyer flask. FDA was added to the suspension to reach the final concentration of 10µg/ml. They were incubated at 30°C on a rotary shaker (120rpm) for 1 hour. After 15min intervals, 1ml of the suspension was taken and placed in microcentrifuge tubes and acetone (50% V/V) was added to stop the reaction (Schnürer & Rosswall, 1982). Then, it was centrifuged for 1min in a microcentrifuge (6000rpm) to remove solid particles. The amount of released fluorescein was measured at absorbance of 494 (A_{494}) and results were expressed as mg of fluorescein, which was released from FDA within 60minutes (Kerem *et al*, 1992; Swisher & Carroll, 1980; Schnürer & Rosswall, 1982).

5.2.4. Liquid-state fermentation

5.2.4.1. Medium and fungus

Highley's basal liquid medium (Highley, 1973) was prepared as follow and standard white rot fungus *Trametes versicolor* was grown on it.

- NH_4NO_3	2 g
- KH_2PO_4	2 g
- $MgSO_4 . 7H_2O$	0.5 g
- $CaCl_2 . 2H_2O$	0.1 g
- H3BO4	0.57 mg
- $MnCl_2 . 4H_2O$	0.036 mg
- $ZnSO_4 . 7H_2O$	0.31 mg
- CuSO4 . $5H_2O$	0.039 mg
-$(NH_4)_6Mo_7O_{24} . 4 H_2O$	0.018 mg
- $FeSO_4 . 7H_2O$	0.015 mg
- Thyamine-3-hydrochloride	0.001 g

(L-asparagine was used instead of thyamine-3-hydrochloride as a vitamin source.)

- Glucose (as a carbon source)	2 g

All were weighed based on 1 liter of distilled water.

Three fungal pellets were punched out from ME fungal plates and placed in each Erlenmeyer flasks (500ml) with 50ml of above medium as pre-cultures and incubated at 23°C and 70% relative humidity. After 1 week, fungal mycelia were homogenized by an Ultra-Thurrax. Six replicates were used for each weight gain and 50ml of medium was placed in the flasks and 10ml of homogenized fungal solution was added to every flask. Sterile autoclaved milled wood was added into the flasks and they were incubated at stationary condition at 23°C and 70% relative humidity.

Sampling was carried out in 2 weeks intervals by taking about 2ml of the culture solution from the flasks.

5.2.4.2. Protein assay

Bicinchoninic Acid (BCA) is a highly sensitive and selective detection reagent for the cuprous cation Cu^{+1}. This protein assay combines the well-known reduction of Cu^{+2} by protein to Cu^{+1} in an alkaline medium with the cuprous Cu^{+1} ion detecting property of BCA. The purple-colored reaction product of this assay is formed by the interaction of two molecules of BCA with one cuprous ion Cu^{+1}. This complex is water soluble and exhibits a strong absorbance at 562 nm, allowing the spectrophotometric quantitation of protein in aqueous solution. Fig. 5-3 represents BCA structure and its reaction. Protein was assayed according to Brown *et al.*, 1989; Smith *et al.* 1985; Wiechelman *et al.*, 1988; SIGMA, 2001.

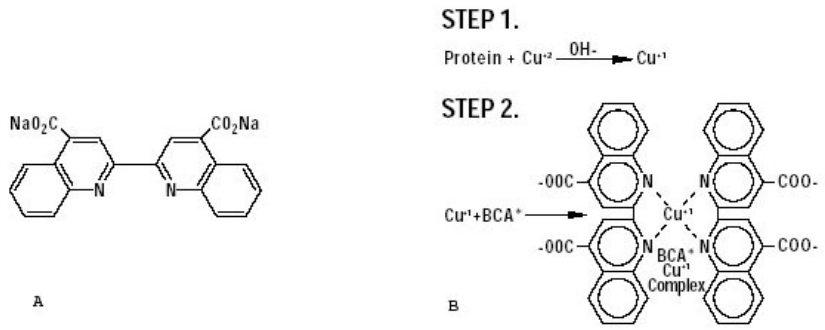

Fig. 5-3. BCA reaction; A: Bicinchoninic acid (BCA), B: Protein react with alkaline copper II to produce copper I. BCA then reacts with copper I to form an intense purple color at 562nm.

Reagents provided:

Reagent A: Bicinchoninic Acid solution (Sigma Chemical Co., Catalogue no. B 9643). Reagent A is a 1000ml solution containing bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1N NaOH (final pH 11.25).

Reagent B: Copper (II) Sulphate Pentahydrate Solution ($CuSO_4.5H_2O$) 4% (w/v)

BCA working reagent: It was prepared by mixing 50 vol. of reagent A with 1 vol. of reagent B.

Protein Standard: Bovine Serum Albumin (BSA) Solution (Sigma Chemical Co., Catalogue no. P 0914). Standard protein curve was prepared with 200, 400, 600, 800 and 1000µg/ml concentrations.

Standard assay: Crude enzyme samples (0.1ml) were combined with BCA working reagent (2ml) and incubated at 60°C for 15 minuets. Absorbance was measured at 562nm.

5.2.4.3. MnP assay

The determination of manganese peroxidase activity was assayed according to Kuwahara *et al.* (1984). Crude enzyme sample (100µl) was combined with 900µl of following reaction solution and incubated at 30°C for 5 minutes. The reaction was stopped with 40µl of 2N NaOH. Oxidation of phenol red was measured at 610nm (A_{610}). The reaction solution without sample was used as a blank.

Reaction solution:

- 0.2% Phenol red	50 µl
- 66 mM Sodium Succinate	300 µl
- 1 mM $MnSO_4$	100 µl
- 0.1 M Lactic acid	250 µl
- 1% Bovine Serum Albumin (BSA)	100 µl
- 1 mM H_2O_2	100 µl

The activity of manganese peroxidase was expressed in units e=4460 $M^{-1}cm^{-1}$.

5.2.4.4. Laccase assay

The laccase enzyme activity was assayed according to Matsumura *et al.* (1986) and modified method by Gutierrez *et al.* (1994). Crude enzyme samples (20µl) were reacted in 160µl of 120mM sodium acetate buffer (pH 5) with 20µl 50mM ABTS [2,2'-Azino-di-3-ethylbenzothiazoline-6-sulfoic acid)] for 5 minutes in multi-well plates. The reaction was measured at 436nm (A_{436}). The laccase activity was expressed in units with e=29300 $M^{-1}cm^{-1}$.

5.2.4.5. Xylanase assay

Substrate: Xylan substrate 1% (w/v) was prepared by dissolving β-1,4-xylan (birch xylan) in de-mineralized water (Yamaguchi & Yoshino, 2001; Yamaguchi, 2001).

Xylanase activity: Reaction was carried out in Ependorf tubes (1.5ml). 400µl of citric acid buffer (preparation method given below) was added to 400µl of the prepared xylan substrate. The mixture was kept at 37°C for few minutes. Crude enzyme (200µl) was afterwards added to the above mixture and agitated vigorously and incubated at 37°C for 15min. The enzyme was denaturated by dipping in boiling water for 5min. The solution was cooled in an ice-bath and then centrifuged at 5000rpm for 15min. Concentration of released sugars in the supernatant was assayed by DNS method (explained below). Liberated sugar (xylose) was subtracted from free sugars in liquid solution. One unit of xylanase activity was taken as the quantity of enzyme that produced 1µmol of xylose in 1min.

5.2.4.6. Endoglucanase (EG) (endo-type β-1,4-gulcanase) activity

Substrate: Carboxymethylcellulose (CMC) 0.5% (w/v) was prepared as substrate by dissolving in de-mineralized water (Yamaguchi & Yoshino, 2001).

Endoglucanase (EG) activity: Reaction was carried out in Ependorf tubes (1.5ml). 400µl of citric acid buffer (preparation method given below) was added to 400µl of the prepared CMC substrate. Crude enzyme (200µl) was afterwards added to the mixture and vortexed. The mixture was incubated at 40°C for 30min. The enzyme was denaturated by dipping in boiling water for 5min. The solution was cooled to room temperature. Concentration of released sugars was assayed by DNS method (explained below). Liberated sugar (glucose) was subtracted from free sugars in liquid solution. One unit of endoglucanase activity was taken as the quantity of enzyme that produced 1µmol of glucose in 1min.

5.2.4.7. CBH (exo-type β-1,4-gulcanase) activity

Substrate: Microcrystalline cellulose was used as substrate for cellulase (CBH) activity. It is not soluble in water or buffer for this purpose 1g of its powder was used as substrate for enzyme reaction.

CBH activity: Cellulose microcrystalline (1g) was placed in the reagent tubes and 1ml citric acid buffer was added. 0.5ml crude enzyme was added to the mixture and agitated vigorously. The mixture was incubated at 40°C for 24h. Agitation of the mixture was repeated for several times. Enzyme was denaturated in boiling water for 5min. The mixture was centrifuged with 2000rpm for 5min and the supernatant (190µl) was sampled and assayed by DNS method (given below). Liberated sugar (glucose) was subtracted from free sugars in liquid solution. One unit of CBH activity is taken as the quantity of enzyme that produced 1µmol of glucose in 1min.

5.2.4.8. DNS assay for reducing sugars

The DNS assay was carried out according to Miller (1959). Reagent solutions and buffers were prepared as follow:

DNS reagent solution:

Reagent A: NaOH (16g) was dissolved in deionized water (200ml) and 3,5-dinitrosalicylic acid (DNS) (10g) was added. The mixture was stirred until the DNS was completely dissolved (The solution should be heated to get complete dissolution).

Reagent B: Sodium potassium tartrate (300g) and metabisulfite (8g) were dissolved in 500ml deionized water.

Both above solutions were combined and diluted to 1 liter.

Buffer: Citric acid monohydrate (11.56g) and sodium phosphate dibasic (Na_2HPO_4) (15.68g) were dissolved in deionized water (1 l) to reach pH 4.5.

Glucose standard: 1g/l solution of glucose was prepared by dissolving 100mg of glucose in 100ml citric acid buffer. Glucose solutions having concentrations of 0.2, 0.4, 0.6, 0.8 and 1g/l were prepared by appropriate dilutions of the standard solution with buffer.

Xylose standard: 1g/l solution of xylose was prepared by dissolving 100mg of xylose in 100ml citric acid buffer. Xylose solutions having concentrations of 0.2, 0.4, 0.6, 0.8 and 1g/l were prepared by appropriate dilutions of the standard solution with buffer.

Standard sugar curves (glucose & xylose): The standard curves for the DNS analysis were prepared by applying the assay procedure given below to each of the standard glucose and xylose solutions (0.2, 0.4, 0.6, 0.8, and 1 g/l) and absorbance was plotted as a function of concentration.

DNS assay Procedure: DNS solution (560µl) was added to 190µl reacted enzyme solution (containing reducing sugars, which were produced by the enzyme during the incubation) or standard sugars and vortexed. The mixture was incubated for 15 minutes in a boiling water bath. Distilled water (750µl) was added to the mixture and vortexed. Reduced sugars were measured at absorbance 550nm (A_{550}). The concentrations were calculated based on the appropriate standard sugar curves.

5.3. Results and discussion

5.3.1. Solid-state fermentation

Mass loss- Figure 5-4 represents mass loss in acetylated and non-acetylated beech wood. The results revealed that increasing weight gain considerably reduced mass loss in the acetylated beech wood (fig. 5-4A). Major loss (60.19%) was determined in the non-acetylated wood after 84 days of incubation and negligible weight loss (1.20%) was measured at the highest weight gain. The results showed that a considerable protection against white rot fungus *T. versicolor* was provided by acetylation above 10.2% weight gain which was about 13 folds less than the non-acetylated one (fig. 5-4B).

Ergosterol assay- Figure 5-5 shows the amount of ergosterol, which was measured in the acetylated and the non-acetylated beech wood. Ergosterol indicates indirectly fungal colonization and its biomass in wood. The results showed that white rot fungus colonized easily in the acetylated and the non-acetylated wood. The colonization was higher in the non-acetylated wood and it was reduced in the acetylated wood at increasing weight gains. During the late incubation period, the amount of ergosterol was raised in the non-acetylated wood and the lowest weight gain (2%), whereas it was not changed in moderately acetylated wood (WPGs 9% and 10.2%) and decreased at the highest weight gain. Low ergosterol content in the acetylated wood indicates lower amount of fungal mass in wood due to insufficient/lack of nutrient in the acetylated wood.

Microcalorimetry- Fungal activity tested with a microcalorimeter is shown in figures 5-6 to 5-8. Figure 5-6 indicates heat production. The heat production was measured for fungal metabolic activities after 14 and 84 days of incubation periods. The results showed that fungal activity was reduced due to the acetylation. The fungus was more active in the non-acetylated wood in comparison with the acetylated ones (fig. 5-6). The activity was increased after 84 days of incubation in the non-acetylated beech wood when it was compared with 14 days of incubation. However, the fungus had less metabolic activity in the acetylated wood after 84 days of incubation comparing to 14 days. Test at the highest weight gain (17%) showed no activity. At the highest weight gains, results showed negative value. The reason could relate to experimental error that could be occurred due to improper sealing of the vials before inserting into measuring channel. Heat could be released due to improper sealing. Energy production during 24 hours of microcalorimetry revealed that total energy production was considerably decreased in the acetylated wood with increasing weight gains, while it was higher in the non-acetylated beech wood (fig. 5-7). Negative energy production was measured at WPG 17% after 84 days of incubation, while it was at least positive after 14 days of incubation. As it was explained above, vials probably were not sealed properly and some energy was escaped from them. Anyhow, no significant energy production was revealed at the highest weight gain (fig. 5-7). Calculated accumulative energy production within 22 hours of its activity (time 2-24

hours) is shown in fig. 5-8. The energy production was decreased when the weigh gain was raised. Energy production was lower at the higher weight gain in comparison with the non-acetylated wood (fig. 5-8). It means that the fungus showed lower activity during 24 hours of microcalorimetry at increasing weight gains.

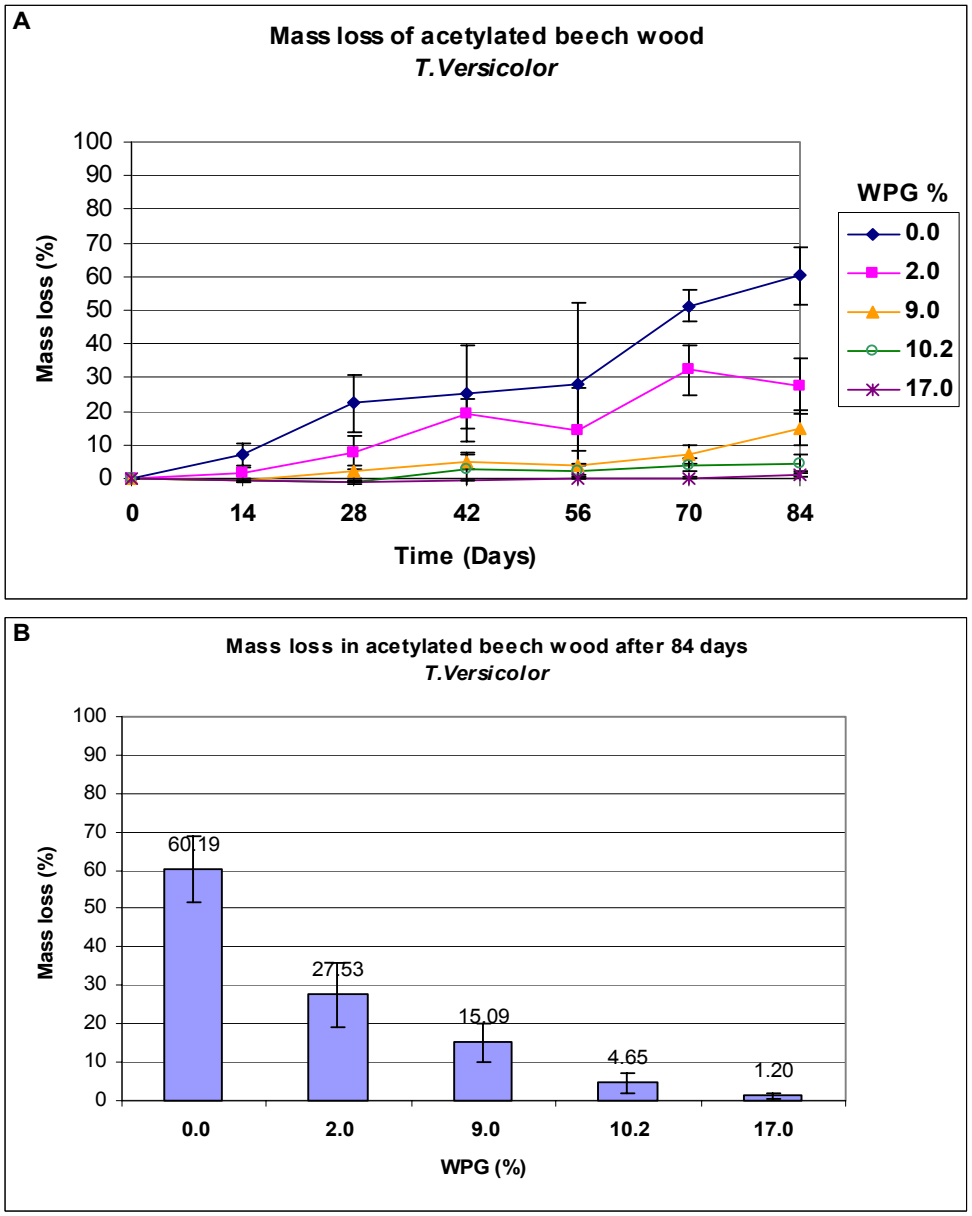

Fig. 5-4. Mass loss in acetylated beech wood; A: During 84 days incubation, B: After 84 days

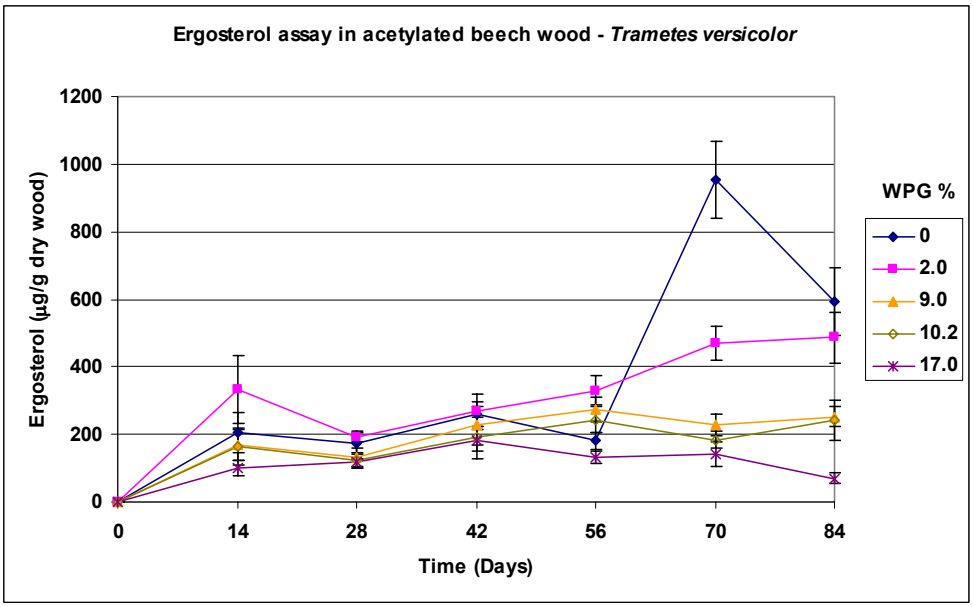

Fig. 5-5. Ergosterol content in acetylated beech wood

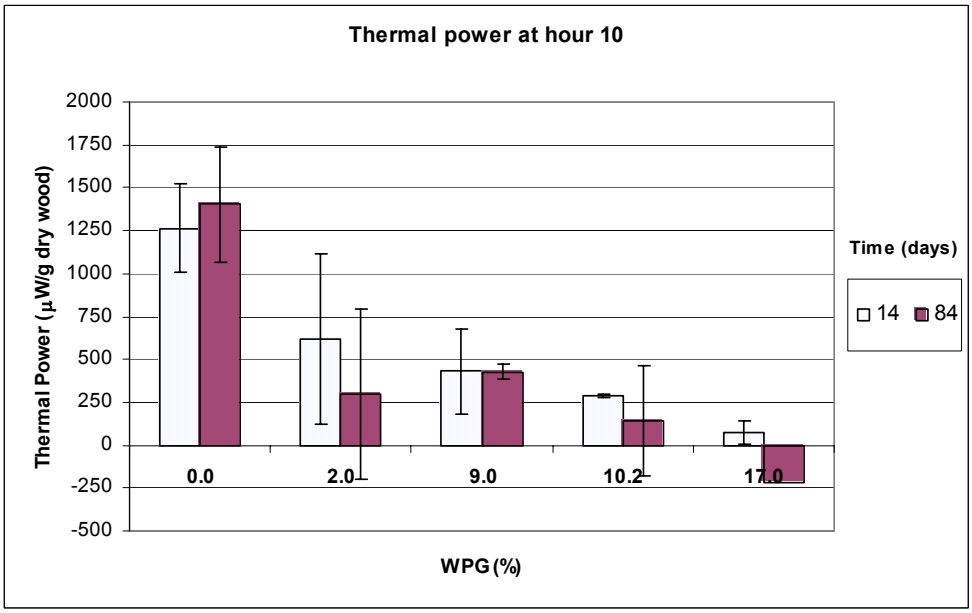

Fig. 5-6. Thermal power in non-acetylated and acetylated beech wood after 10 hours. Thermal power indicates microbial bioactivities in wood.

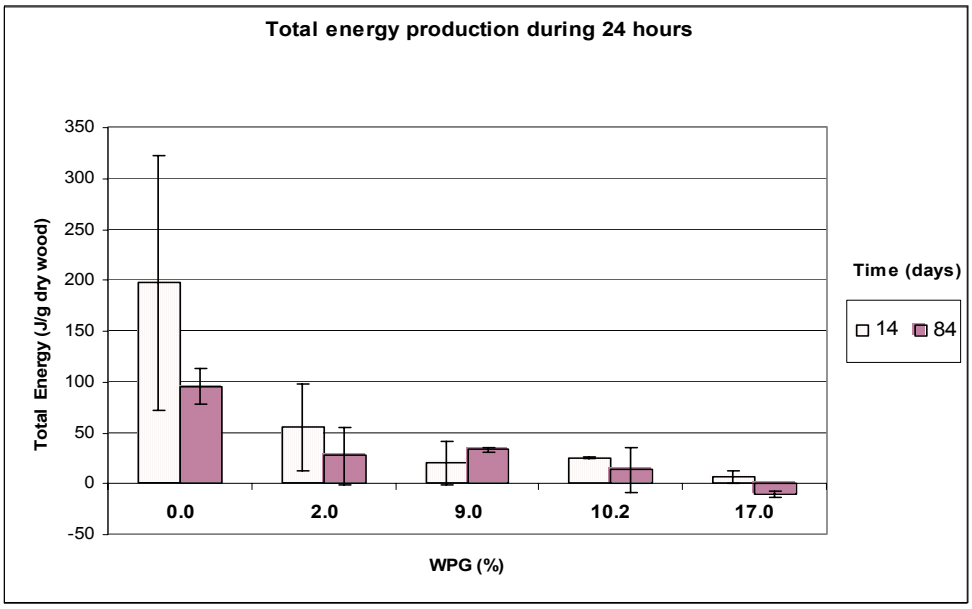

Fig. 5-7. Total energy production in non-acetylated and acetylated beech wood after 24 hours

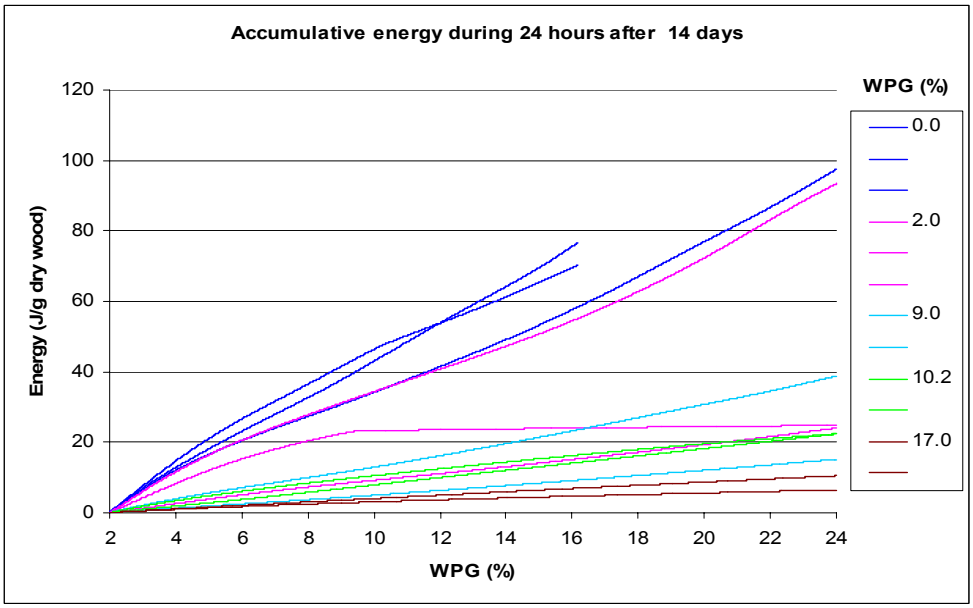

Fig. 5-8. Calculated accumulative energy production in acetylated and non-acetylated beech wood after 14 days

Fluorescein diacetate (FDA) - Fluorescein diacetate is hydrolysed by a number of different enzymes, such as esterases, lipases and proteases. The product of enzymatic conversion is fluorescein, which indicates active fungal biomass. Liberated fluorescein was quantified spectrophotometrically in the acetylated and the non-acetylated beech wood in order to measure bioactive fungal mass in wood (fig. 5-9). FDA hydrolysis showed a low rate of reaction in the acetylated beech wood at increasing weight gains. Higher FDA reaction was determined in the non-acetylated wood and no reaction at the highest weight gain (WPG 17%). However, low FDA reaction rate was measured in moderately acetylated wood (WPGs 9% and 10.2%). It revealed that the FDA reaction was increased during the whole incubation period in the acetylated and the non-acetylated wood except the highest weight gain. Increasing FDA reaction rate indicated raising fungal activities in wood with decreasing WPG.

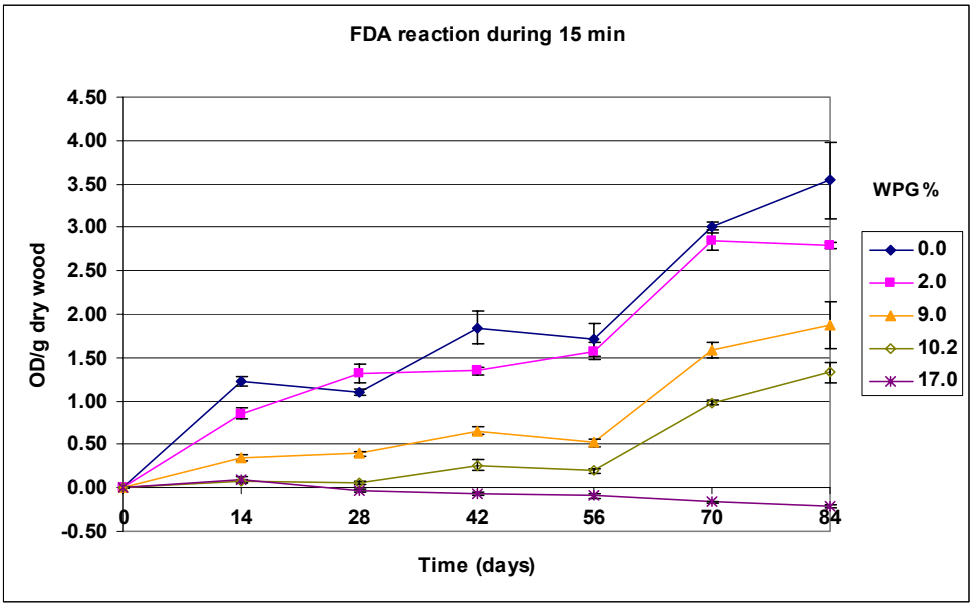

Fig. 5-9. Fluorescein diacetate reaction in acetylated and non-acetylated beech wood

Mass loss determination showed that acetylation provides a considerable bioresistance at weight gains higher than 10%. Mass loss reduced 13 folds lower than the loss in the non-acetylated wood and there was no significant loss at the highest weight gain. Results confirmed other reports. Beckers *et al.* (1994) reported that acetylation protects acetylated beech wood against white rot fungus *Coriolus versicolor* at a WPG of 12.8%. Goldstein *et al.* (1961) and Peterson & Thomas (1978) reported that an acetylation level of 17% weight gain is adequate to control fungal decay and 10% was the efficient level to achieve a good protection. It was also reported that acetylated pine and eucalypt flakeboards at weight gains of 16-18% prevented against white- and brown-rot fungi (Okino *et al.*, 1998). Takahshi *et al.* (1989a&b) showed that weight gains between 10-15% had striking decrease effect on brown rot fungus *Tyromyces palustris* and preventing at WPG 20%, while the striking decrease level for *Coriolus versicolor* was between 12-15% and prevention at WPG 20% in non-durable wood species. Kalnins (1982) tested acetylated woods in soil for 12 weeks using two brown rot fungi (*Gloeophyllum trabeum* and *Lentinus lepideus*) and a white rot fungus (*Coriolus versicolor*) and reported that acetylation gives a very good resistance against these fungi when the weight gain reached above 15%. Ohkoshi *et al.* (1999) showed that weight loss due to white rot fungus *Coriolus versicolor* decreased slowly with the increased WPG and reached zero at about 12% of weight gain. Suttie *et al.* (1997 & 1998) showed that white rot fungus *C. versicolor* was unable to attack acetylated wood at weight gain of 18.6%.

Decreasing ergosterol amount in the acetylated wood in comparison with the non-acetylated one indicates that the fungus autolyzed its mycelia due to insufficient/lack of nutrients in the acetylated wood to save energy and live during the incubation period (fig. 5-5). However, microcalorimetry revealed that fungus could not produce any metabolic heat and energy to indicate that it is alive at the highest weight

gain (17%), while during the first weeks of incubation, it had produced metabolic heat (fig. 5-6 to 5-8) indicating its active metabolism. The metabolic activity was lower than the non-acetylated wood and also low weight gains. Comparison between heat production during 14 and 84 days of incubation indicates that fungus had active metabolism during the first period of incubation in all acetylated wood and it was decreased at the end of incubation. However, it was increased in the non-acetylated beech wood (fig. 5-6). The explanation of this phenomenon could be in-/low-accessibility of cell wall polymers for fungal mycelia in the acetylated wood at low and moderate degrees of the acetylation, which could be blocked for enzymatic activities due to high degree of the acetylation at WPG 17%. High thermal power in the non-acetylated wood could relate to higher activity of white rot fungus due to accessibility of cell wall polymers for hydrolyzing enzymes. Same phenomenon was observed for energy production in all types of wood even the non-acetylated one (fig. 5-7). It indicates that fungus had sufficient activity during the first days of the incubation and it was decreased to the end of the incubation period. Total energy production shows that white rot fungus loses its activity with increasing weight gains (fig. 5-8). Active fungal metabolism produces higher amount of energy in the non-acetylated wood, while it decreases in the acetylated wood. Insignificant fungal activity at the highest weight gain indicates that white rot fungus was ceased its activity or might be dead due to blocked cell wall polymers and consequently starvation.

FDA reaction showed that enzymatic activity was reduced due to the acetylation (fig. 5-9). No activity at the highest weight gain (17%) reveals that fungus had no metabolic activity in the highly acetylated wood. Increasing FDA reaction in the non-acetylated wood and the lowest weight gain (2%) indicates that fungal active mass was increased in those woods and their enzymes could access to cell wall polymers. However, FDA hydrolysis was low in moderately acetylated wood compared with the non-acetylated wood and the lowest weight gain. Overall FDA reaction rate was increased during the incubation period, except the highest weight gains. The variation of the FDA reaction is very similar to that of ergosterol. Both indicate increasing fungal mass in all types of wood except the highest weight gain. Presented results indicate that both methods (ergosterol assay and FDA hydrolysis) monitor fungal activity in all types of woods.

5.3.2. Liquid-state fermentation

Since fungal growth on acetylated wood in condition of liquid state-fermentation was under question, this part of research was conducted to test whether fungal growth could give good results or not. Improper growth of fungus was obtained and some variation in the results was achieved. However, the results are presented as follow.

Total protein- Based on BCA assay, total protein was assayed in the acetylated and the non-acetylated beech wood (fig. 5-10). Enzymes have protein structure and here these results indicate that general amount of released enzymes were high in the non-acetylated beech wood and it was low in the acetylated wood. Between the acetylated wood, higher amount of protein was measured in low acetylation. Time course study showed raising amount of protein in all types of wood.

Laccase activity- Laccase activity increased at raised weight gains (fig. 5-11). Higher enzymatic activity was assayed at WPG 17% and the lowest activity was measured in the non-acetylated beech wood.

MnPase activity- Figure 5-12 represents that *T. versicolor* showed no MnP activity in all types of wood.

Xylanase activity- Liberated xylose by xylanase enzyme was assayed in the non-acetylated and the acetylated beech wood. The results are shown in fig. 5-13. A time course study showed that xylanase activity was slightly higher in moderately acetylated woods (WPGs 9% & 10.2%) than that in the non-acetylated beech wood. And slight activity was shown at the lowest weight gain (2%) and no activity at the highest weight gain (17%). At later stage of incubation, the non-acetylated wood showed higher activity in comparison with all acetylated woods.

EG (endo-type β-1,4-glucanase) activity- EG assay showed no significant activity in all types of wood (fig. 5-14).

CBH (exo-type β-1,4-glucanase) activity- Figure 5-14 represents CBHase activity in the acetylated and the non-acetylated beech wood. A time course study showed higher activity in the non-acetylated wood in comparison with the acetylated beech wood. No CBHase activity was measured at the highest WPG (17%).

Total protein measurements showed that white rot fungus *T. versicolor* could release lower amount of enzymes in the acetylated wood. The amount of enzyme was influenced by the acetylation and decreased at raising weight gains (fig. 5-10). It reveals that the acetylation of wood affects biological activity of white rot fungus and it can not produce wood-degrading enzymes to attack wood. However this fungus could easily colonize in wood during early period of incubation (as ergosterol assay and FDA analysis have revealed it before) and its metabolic activity reduced due to inaccessibility of cell wall polymers in the acetylated wood (as microcalorimetry showed it before).

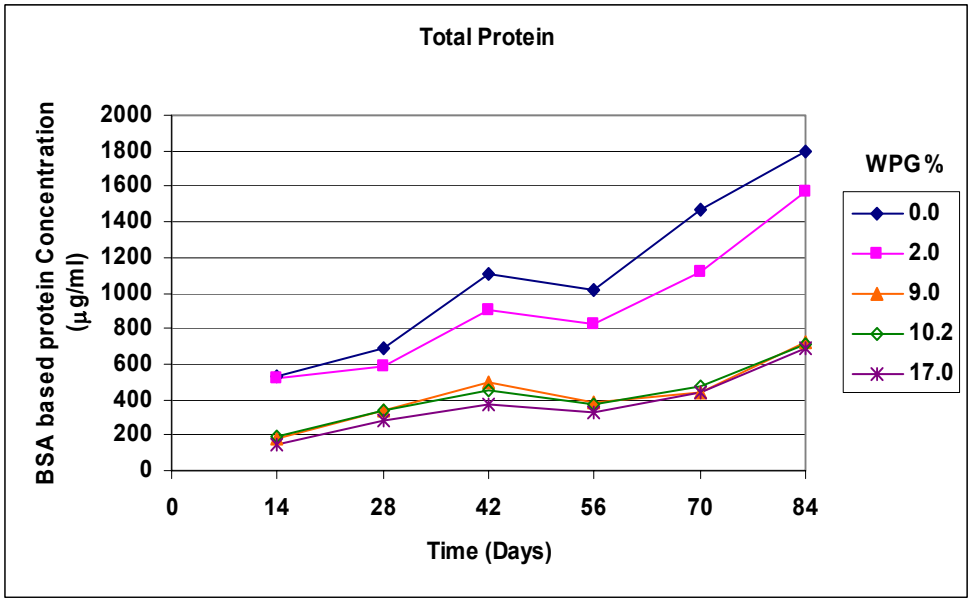

Fig. 5-10. Total protein in acetylated and non-acetylated beech wood

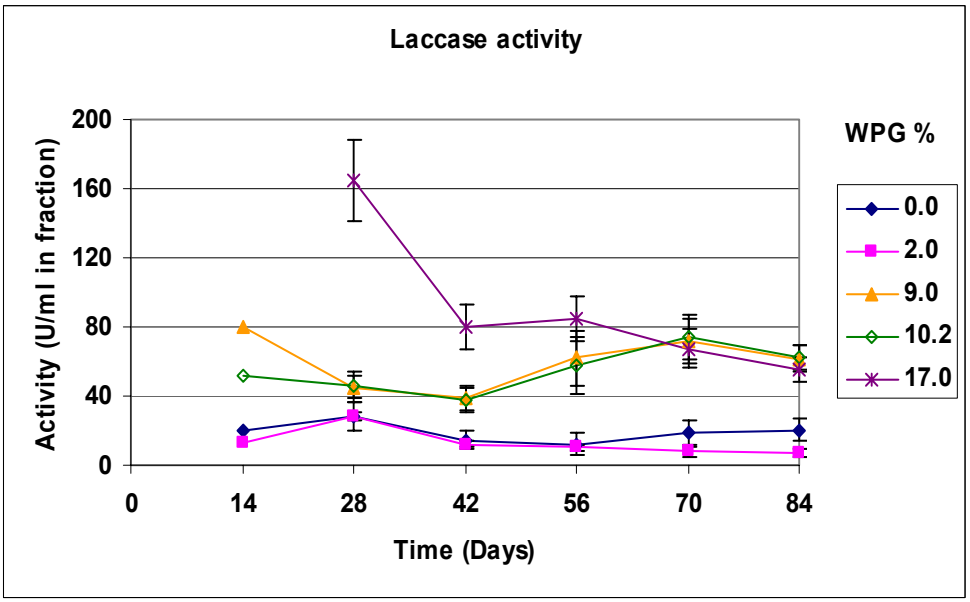

Fig. 5-11. Laccase activity in acetylated and non-acetylated beech wood

In spite of low protein, laccase showed higher activity in the acetylated wood and it was more active in the acetylated wood than that of the non-acetylated one. This result provides a contradiction when it is compared with total protein assay. The reason for higher laccase activity is unknown for us. According to Matsumura *et al.* (1986), ABTS [2,2'–Azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)] combines with phenol derivatives, hydroxybenzoic acids, in the presence of laccase to form coloured compounds which shows stronger absorption (fig. 5-16). This absorption could be measured by spectrophotometry. Lignin structures could probably influence ABTS reaction. However, no logic interpretation could be expressed for the measured higher ABTS reaction in the acetylated wood.

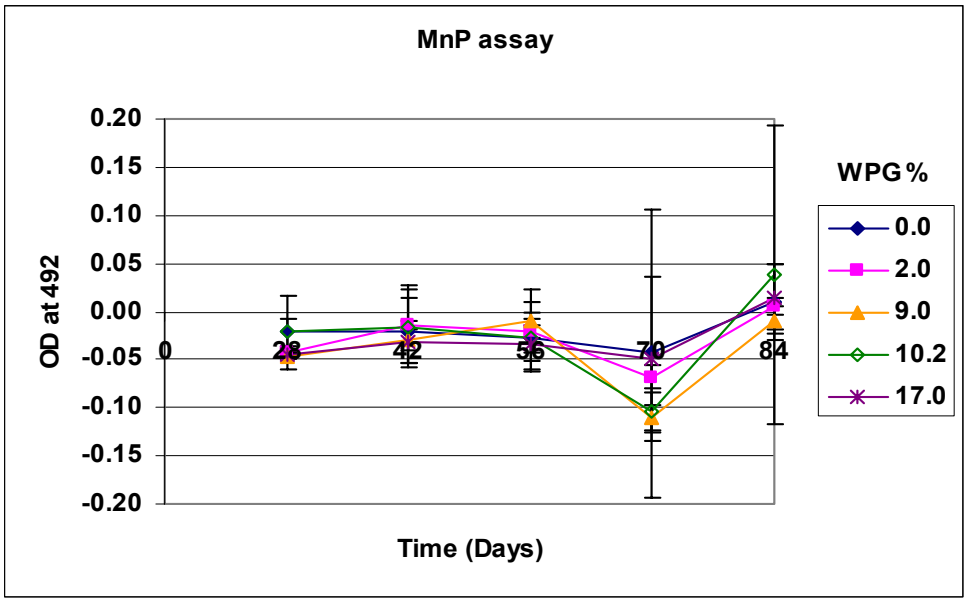

Fig. 5-12. Manganese peroxidase activity in acetylated and non-acetylated beech wood

Insignificant MnPase activity in all types of wood, even the non-acetylated one, reveals that the fungus *T. versicolor* could/did not probably produce any MnP enzymes to degrade wood in presence of laccase. It was suggested that MnPase oxidises Mn^{2+} to Mn^{3+} and this product is responsible for all oxidations of the substrates. This complex oxidises probably lignin in wood after diffusion (Eriksson *et al.*, 1990).

Study on xylanase activity showed variable results in all types of wood. This variation could be related to different mode of actions of white rot fungus *T. versicolor* regarding to attacked wood type. It was reported that several types of enzymes involve in degradation of hemicellulose xylan in wood due to its branched hetero-polysaccharide structure. A complete degradation of a branched acetyl xylan requires a concerted action of several hydrolytic enzymes, i.e. endo-1,4-β-xylanase, β-xylosidase, α-glucuronosidase and acetylestrase or acetylxylanesterase (Eriksson *et al.*, 1990). Concerning this report, the fungus probably produces more enzymes to attack acetylated xylan. Therefore, during the assays more types of xylanase enzymes could probably involved in reaction and showed higher activity in the acetylated wood than that in the non-acetylated and the lowest weight gain. Insignificant reaction at the highest weight gain could be related to less fungal mass due to blocked structure of xylan and cell wall polymers. Another reason for different xylanase activity in these woods could be related to the used substrate. As reported, there are many factors causing variation in the determination of xylanase and also xylosidase activity. For example, dilution of enzyme prior to assay is one of the technical problem in assaying method. Also determination of xylanase was influenced by the type of substrate, which affects different xylanase involving enzymes (Eriksson *et al.*, 1990).

The insignificant activity of EG (endo-type β-1,4-glucanase) could be interpreted as inability of white rot fungus in producing of cellulose hydrolyzing enzymes. However, concerning slight activity in the non-acetylated wood, probably carboxymethycellulose was not suitable type of substrate, which was used in the study. Reports indicated that lack of the specific substrate and standardization of activity determinations are

still complicated problems in cellulase enzymes assays. Therefore no fruitful results of this study probably relate to the selected substrate. Similar problem was also reported for CBH (exo-type β-1,4-glucanase) activity, because, no standard substrate is still available for a direct and specific measurement of exo-type β-1,4-glucanase activity in a mixture of cellulolytic enzymes (Eriksson *et al.*, 1990). Therefore, different reactions of cellulases in this study probably relates to the type of substrate.

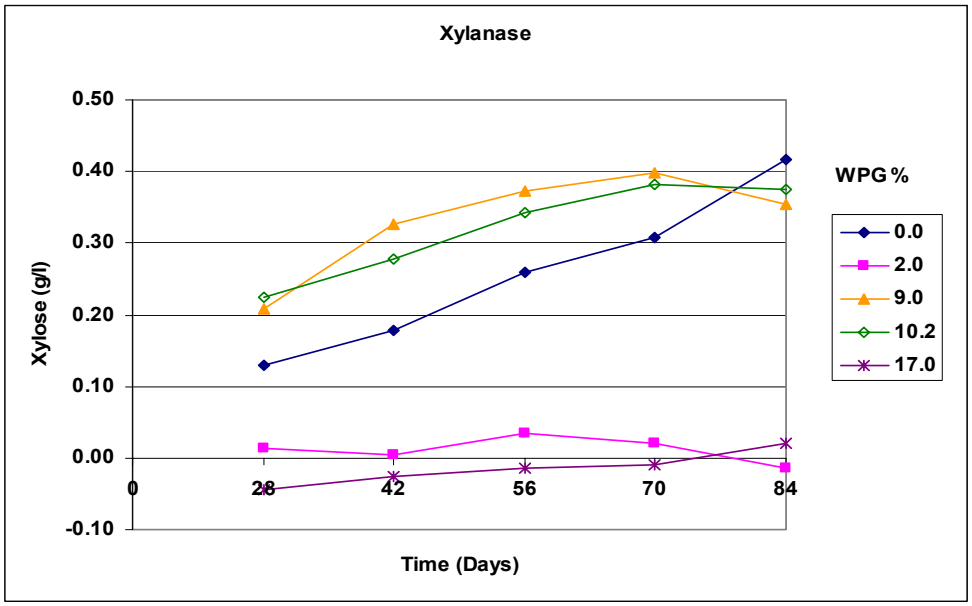

Fig. 5-13. Xylanase activity in acetylated and non-acetylated beech wood

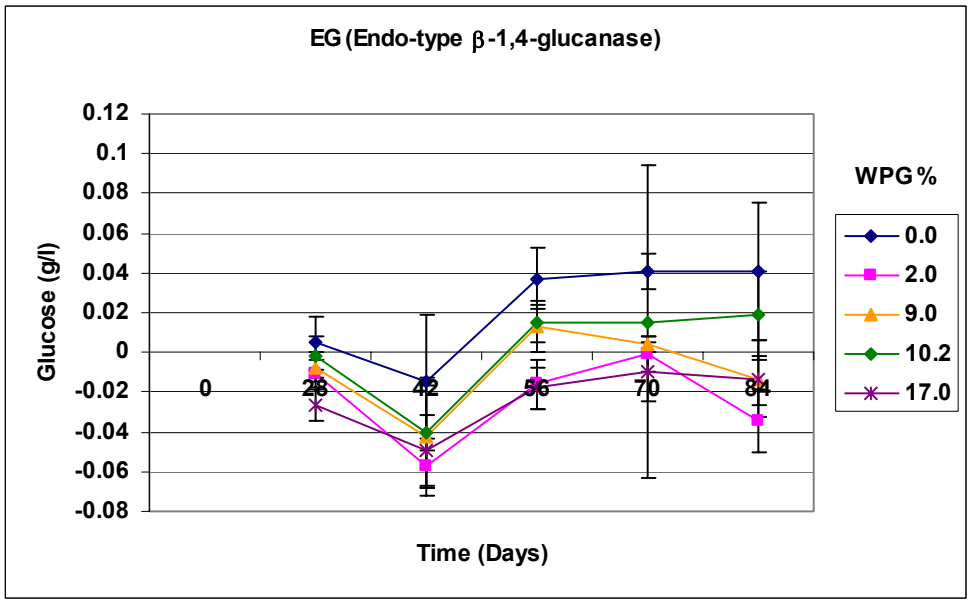

Fig. 5-14. EG (endo-type β-1,4-glucanase) activity in acetylated and non-acetylated beech wood

Concerning the overall obtained variable results in liquid state-fermentation, another explanation could be changing pH of liquid media due to acetylated samples. As shown, pH of media influences strongly fungal activity (Zabel & Morrel, 1992) and probably different pH of acetylated samples influenced the pH in media. Another reason for this complication is using the liquid medium. Regarding the explanation

above, thyamine-3-hydrochloride was substituted with L-asparagine in this study. Thus the substitution probably influenced generally the fungal growth.

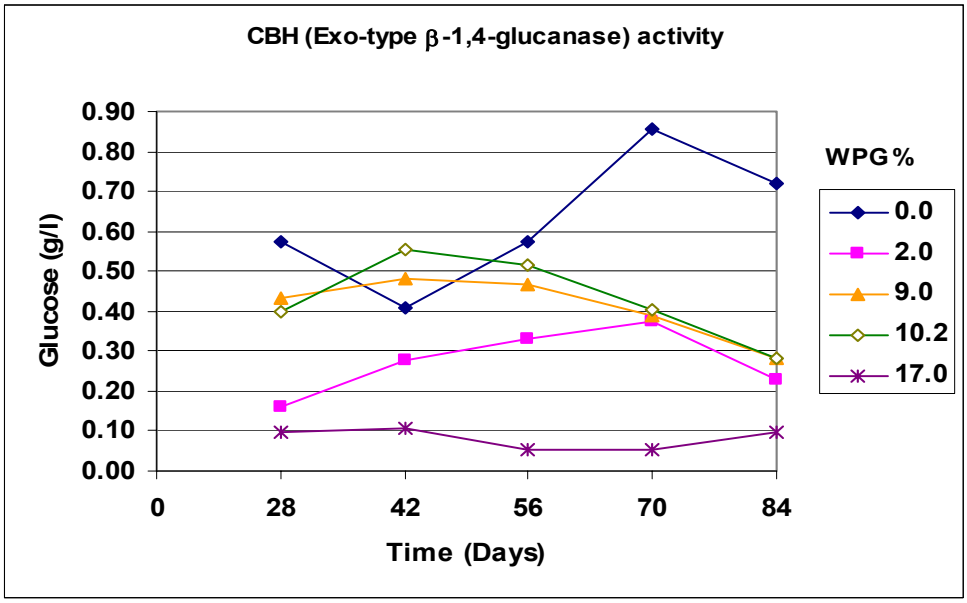

Fig. 5-15. CBH (exo-type β-1,4-glucanase) activity in acetylated and non-acetylated beech wood

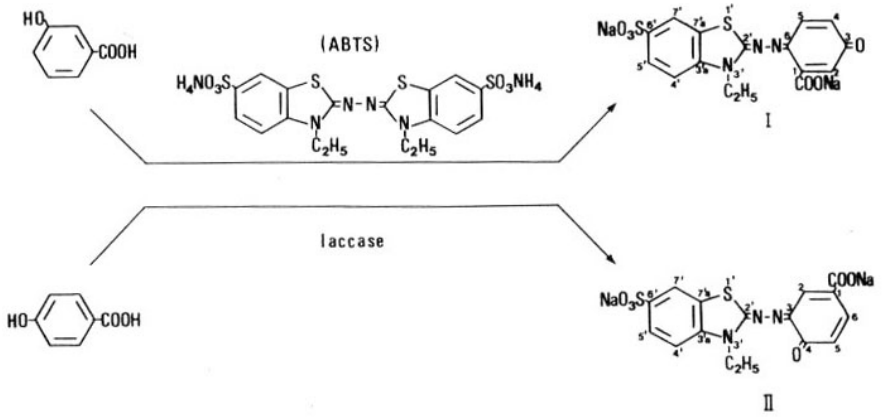

Fig. 5-16. ABTS reaction

5.4. Conclusion

In general, the results indicated that acetylation provides a good protection in beech wood and reduces mass loss in wood. White rot fungus colonized the acetylated and the non-acetylated wood. However the fungus loses its power to decay acetylated wood due to inaccessibility of cell wall polymers for fungal enzymes and it cannot survive there and finally the fungus might die or lives in common form and a part of dead/inactive mycelia remains in cell lumina.

All explanations above show that there are still many difficulties in study of liquid-state fermentation and caring of all factors needs many detailed studies that should be investigated separately, because proper growth of fungi is crucial. Therefore repetition of this part of research is suggested to solve many technical problems to project a new light for future studies about the acetylation of wood.

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Chapter 6

General Discussion

6.1. Acetylation level and protection mechanism

Soil bed tests revealed inhibitory effect of the acetylation on soil microorganisms. Acetylation at higher than 8% of WPG in beech and 10% of WPG in pine considerably influenced biological attack in wood, however higher WPGs are required for a total protection (17% and 19% of weight gains in beech and pine respectively). The use of dynamic modulus of elasticity (MOE_{dyn}) showed similar results as mass loss. The loss of MOE_{dyn} in wood was decreased at increasing weight gains. Similarly, MOE_{dyn} was considerably decreased at higher than 8% WPG in beech and 10% WPG in pine wood. No loss in MOE_{dyn} was revealed at the highest weight gains (17% WPG in beech and 19% WPG in pine).

Our studies showed slight raises in mass in the highly acetylated woods. It is probably due to colonization of hyphae in cell lumina without any attack, which was shown by microscopical studies. Wood mass is influenced by the hyphal mass and any changes in wood mass affected MOE_{dyn}. Machek *et al.* (1997, 1998a&b, 2001) used a vibration technique to determine MOE in attacked wood and showed that the vibration method is a more sensitive indicator of wood decay than mass loss. They reported that MOE_{dyn} losses for non-durable and slightly durable species (*e.g.* beech, poplar and elm) ranged from 77-86% and their mass losses after 12 weeks of exposure ranged from 35-40% (Machek *et al.*, 1998a).

Solid fermentation tests by using standard white rot fungus (*Trametes versicolor*) also showed that acetylation decreased weight loss in beech wood at increased weight gains. Major loss by white rot fungus was determined in non-acetylated wood and at low degree of the acetylation and no signification loss was measured at the highest weight gain. The tests revealed that acetylation decreased considerably the weight loss due to white rot decay in beech wood at above 10.2% of weight gains.

Our investigation confirmed previous reports of other authors. Study on soil bed tested acetylated beech, pine and poplar showed inhibitory effect of the acetylation on soft rot attack at WPGs of about 10, 10.7 and 14.4% respectively (Beckers & Militz, 1994). It has also been reported that for poplar a WPG of 11.2% and for beech a WPG of 12.8% was required to achieve the same results in field soil tests (Beckers *et al.*, 1995). Similar results have also been reported for beech and pine wood against soft rot fungi. Weight gains above 8% for beech and 10% for pine had considerable influences against soft rot fungi in soil test (Mohebby & Militz, 2002). Larsson *et al.* (1997) tested the acetylated wood in ground contact samples and reported that acetylation had a major impact on the fungal resistance of wood. The resistance of the acetylated wood against fungal attack at WPG of about 20% is at the same range as that of wood with higher retention of preservatives (copper-chromium containing). It was also reported that fungal decay in mini-stakes, which were exposed to unsterile soils in laboratory condition were significantly decreased at acetyl content of 15.1%. The acetyl content of 18.5% prevented most attack by tunneling bacteria (Larsson *et al.,* 2000). Takahashi *et al.* (1989) reported an enhancement of decay resistance by acetylation. Takahshi *et al.* (1989a&b) determined weight loss in acetylated wood and showed that acetylation between weight gains 10-15% had striking decrease effect on brown rot fungus *Tyromyces palustris* and preventing at WPG 20%, while the striking decrease level for *Coriolus versicolor* was between 12-15% and prevented decay at WPG 20% in non-durable wood species. Another report showed that the protection level was above 6% in acetylated wood (Kumar, 1994). Nilsson *et al.* (1988) also tested acetylated particleboards in soil in different cellars and reported that at WPG of about 15%, no attack by microorganisms was observed after 12 month. Ohkoshi *et al.* (1999) reported an inhibitory effect of the acetylation at WPG of more than 10% on the fungal degradation. They showed that the decay by brown rot fungus (*Tyromyces palustris*) became inhibited at a weight percent gain higher than 10% and the mass loss due to decay became zero at a WPG more than 20%, while the weight loss due to white rot decay (*Coriolus versicolor*) decreased slowly with the increase in WPG and reached zero at about 12%. Militz (1991) also studied the effect of the acetylation at weight gain 20.8% in beech wood and reported that brown rot fungi (*Gloeophyllum trabeum*, *Poria placenta* and *Coniophora puteana*) failed to attack the acetylated wood. Resistance of sweetgum was tested by Kalnins (1982) in soil for 12 weeks using two brown rot fungi (*Gloeophyllum trabeum* and *Lentinus lepideus*) and a white rot fungus (*Coriolus versicolor*). Tests showed a very good resistance against those fungi when the weight gain reached above 15%. Okino *et al.* (1998) reported that acetylated pine and

eucalypt flakeboards at weight gains 16-18% prevented against white- and brown-rot fungi. Several authors have reported that an acetylation level of 17% weight gain is adequate to control fungal decay and 10% was the efficient level to achieve a good protection (Goldstein *et al.*, 1961; Peterson & Thomas, 1978). Suttie *et al.* (1997 & 1998) showed that white rot fungus *C. versicolor* was unable to attack acetylated wood at weight gain of 18.6%.

6.2. Chemical aspects of protection in acetylated wood

Reports have shown that soft rot fungi remove α-cellulose, hemicelluloses and lignin from wood. It was indicated that wood polysaccharides are degraded in preference to lignin (Savory & Pinion, 1958; Levi & Preston, 1965; Seifert, 1966; Nilsson *et al.*, 1989). Levi and Preston (1965) also analyzed removal of lignin and methoxyl groups in lignin by soft rot fungi. The rate of removal for methoxyl groups was considerably higher than that of lignin itself. Also soft rot fungi can modify lignin during decay. A study on syringyl : guaiacyl (S:G) ratios of the residual lignin in birch wood decayed by several types of ascomycetous fungi revealed that the syringyl-propane units are being preferentially degraded (Nilsson *et al.*, 1989; Nilsson *et al.*, 1988). Since lignin of hardwoods have more methoxyl groups and higher S:G ratios than softwoods (Fengel & Wegner, 1989), soft rot fungi decay lignin in beech more preferential than in pine also at higher WPGs. Butcher and Nilsson (1982) also demonstrated that high lignin timbers are less prone to soft rot than low lignin timbers. The amount of lignin in beech is lower than pine wood (22.70% and 27-28%, respectively) (Butcher and Nilsson, 1982; Nilsson *et al.*, 1988). Our results indicate the amount of lignin for beech 21% and Scots pine 30% (the differences between the reports could be due to different analytical methods). Therefore the higher durability of non-acetylated pine wood in comparison with beech wood probably related to higher lignin content in pine and more guaiacyl units in its lignin.

Rosenqvist (2001) reported that uniform distribution of acetyl groups due to the acetylation in cell walls occurs at higher weight gains. Ohkoshi *et al.* (1997a&b) studied the acetyl groups in lignin, cellulose and hemicelluloses and reported that substitution of hydroxyl groups in amorphous regions of cellulose begins at WPG 5.7% and all groups are substituted at WPGs above 20%. We can conclude from our own results that the differences in removal of cell wall components by soil microorganisms could be due to the differences in degree of substitution of the hydroxyl groups with the acetyl groups in both species during the acetylation, and also differences in chemical structure of the lignin itself (which is more methoxylated in beech than in pine). Levi and Preston (1965) also reported an increase in the average degree of polymerization (DP) of the cellulose in beech wood attacked by *Chaetomium globosum*. Crystalline cellulose remains intact due to a preferential degradation of the amorphous regions in cellulose. Regarding this report, we could conclude that soft rot fungi have probably no capability to degrade amorphous regions of cellulose due to the acetylation and it gives a good protection to cellulose against soft rot fungi. On the other hands, soil fungi remove methoxyl group preferentially. Therefore, more removal of beech lignin in comparison with pine probably related to more methoxyl groups in syringyl lignin (beech) than guaiacyl lignin (pine). On the other hands, acetylation does not influence methoxyl groups in lignin.

Any slight raise of chemical components at the highest WPGs (in comparison with untreated controls) could likely relate to the existence of hyphae, which are presented in cell lumina of highly acetylated wood without causing any attack. Mycelial walls contain polysaccharides (Burnett & Trinci, 1979). Consequently the higher amount of cellulose in highly acetylated wood probably relates to presence of hyphae in cell lumina.

Studies on IR spectra from soil exposed acetylated and non-acetylated wood showed a decrease in wave numbers 1737-1728cm^{-1}, which indicate C=O stretching in hemicelluloses. Any decrease could relate to cleaved acetyl groups in hemicelluloses. Increase in wave numbers 1595 cm^{-1}in beech wood and 1666-1643 cm^{-1} in pine wood, which are also related to stretching of OH groups in polysaccharides, indicate attack on glycosidic linkages. OH groups being produced due to opening of those linkages. Any decrease in wave numbers 1506-1502 cm^{-1} (indicating C=C stretching in lignin) in non-acetylated and moderately acetylated beech wood except the highest WPG in beech wood, indicates that lignin is attacked due to enzymatic activities. IR spectroscopy showed a decrease in wave numbers 1370-1369 cm^{-1} (representing C-H deformation of CH_3 in acetyl groups in lignin) in non-acetylated and moderately acetylated wood except the highest weight gain, which indicates cleavage of β-O-4 linkages in lignin due to ligninolytic enzymes. Ligninolytic activities of soft rot fungi have been reported by some authors (Levi & Preston, 1965; Blanchette *et al.*, 1990; Nilsson *et al.*, 1989; Durán *et al.*, 1987 and Tanaka *et al.*, 2000).

6.3. Microscopical evaluation of microbial attack

Microscopy on samples from field soil trails (The Netherlands) revealed that soft rot and white rot decay were occurred in non-acetylated and acetylated wood in different degrees after 5 years exposure to the soil. While no brown rot decay was observed in these woods. Bacteria were detected in all types of wood. They generally cooperate with soft rot fungi to degrade non-acetylated wood or limit themselves to pits at the higher degrees of the acetylation.

Observation of soft and white rot decay in same samples probably relates to soil moisture, which accelerates due to climatological variation during a year. Changing soil moisture affects the oxygen level in the soil. When the soil is saturated, there is a limitation of oxygen, which restricts aerobic microorganisms and permits anaerobic or facultitative microorganisms. Consequently, variation of moisture in the soil affects the moisture content in planted wood samples in the field soil and changing the moisture in the wood samples influences the oxygen level in wood stakes. Reports indicate that oxygen is a crucial factor that can affect strongly activities of microorganisms in wood (Zabel & Morrell, 1992).

Reports show that soft rot fungi are active in a wide range of moisture contents, from relatively dry wood (Daniel & Nilsson, 1998) to saturated condition (Singh & Kim, 1997; Machek *et al.*, 1997; Zabel & Morrell, 1992), whereas optimal wood moisture level for most decay fungi (basidiomycetes) lies between 40 and 80%. White rot fungi require more moisture than brown rot fungi to achieve optimal wood weight loss (Zabel & Morrell, 1992). Moisture content around fiber saturation point (28-30%) is required for brown rot fungi (Ritsckoff, 1996). Soft rot fungi can also decay wood in a wide range of temperature from 0°C to about 60°C (Daniel and Nilsson, 1998), while the optimum temperature for basidiomycetes is between 24-32°C (Zabel & Morrell, 1992).

Kazemi *et al* (1998 & 1999) reported that in soft rot fungus *Chaetomium globosum* increasing the moisture content in wood generally causes weight losses to be increased on beech and Scots pine wood. Reduced oxygen level appears to cause much smaller reduction in decay by *C. globosum* than with the basidiomycetes (*Coniophora puteana* and *Coriolus versicolor*). Oxygen plays a clear role on white and brown rot decay processes.

Soft rot fungi have good capability to adopt to accelerated soil moisture and temperature to decay wood during whole period of a year, while white rot fungi can decay wood during a short period when the temperature and moisture is in optimal level, e.g. summer, end of spring and early autumn seasons. It seems soil moisture in the used field for research is higher than the optima for brown rot fungi to be activate in wood. Therefore, no brown rot decay has been distinguished in wood stakes due to high moisture content and low level of oxygen. Considering the results, rot fungi were more active in hardwood species, beech and poplar, than in Scots pine wood. The reason is probably related to lignin type in these woods. Levi & Preston (1965) reported that soft rot fungi decay hardwoods more preferable than softwoods, because hardwood lignin (syringyl type) is more methoxylated than softwood lignin (guaiacyl type) (Levi & Preston, 1965).

On the other hands, soil pH is another reason, which can influence fungal growth. Reports show that brown rot fungi prefer lower pH and white rot fungi grew at higher than the optimal pH for those fungi. Soft rot fungi are also active at pH close to neutral (Zabel & Morrel, 1992). Probably in the test field, soil had not optimal pH for brown rot fungi and there was optimal condition for soft- and white rot fungal growth. Therefore, it could be concluded that soil type, moisture variation, oxygen level and wood species were the main reasons that influenced microbial growth in acetylated wood samples from the test field.

Successional changes in colonizing of wood could probably be considered as another reason for different microbial degradation that was occurred in the wood stakes. Käärik (1975) reported successional changes in the colonizing microorganisms from the initial phase of bacteria, moulds, blue stain and soft rot fungi to the take-over by basidiomyceteous fungi and also successive changes in the attacking decay fungi.

At the highest WPGs fungal hyphae can colonize into cell lumina and do not decay wood considerably. The observations also confirm findings of Kumar (1994) that decay hyphae limit themselves to parenchyma cells and tracheid lumina and fail to attack the acetylated cell walls. The slight development of decay in the acetylated wood comparing the non-acetylated wood proved a very good protection of wood against soil microorganisms due to the acetylation after 7 years period.

Concerning the microscopical observations on basidiomycete tests, it can be concluded that hyphae of both standard fungi (*Trametes versicolor* and *Poria placenta*) can easily colonize in cell lumina and ray cells. Both fungi use open ways to penetrate into wood tissue, preferentially vessel lumina and rays at earlier stage and then penetrate into fiber cell lumina through inter-fiber or cross-field pits between rays and fibers. Rays in the non-acetylated beech wood and probably at low weight gains have enough nutrients for hyphal growth. However, in moderately and highly acetylated wood, those nutrients were probably removed during the acetylation process and no or insufficient nutrients were remained for fungal growth in ray cells. Lack of the nutrients and protection of the ray cell walls against fungi due to the acetylation could be the main reasons for slight or no decay in the ray tissues of the moderately and highly acetylated wood.

Any change in cell wall birefringence represents attack on crystalline cellulose (Anagnost, 1998 & Wilcox, 1993a&b, Wilcox, 1968). Our observations showed that in the non-acetylated wood and at the lowest weight gain, the loss of birefringence occurs in cell walls due to fungal attack at early stage of decay. However, acetylated wood cell walls have lost their birefringence before any attack at weight gains above 8.84% in beech and 9.97% in pine wood. This indicates that crystalline cellulose was probably influenced due to the acetylation process. Perhaps substitutions of hydroxyl groups in crystalline cellulose with acetyl groups during the acetylation could influence the polarization behavior of crystalline cellulose under polarized light and sequentially the birefringence property of acetylated cell walls was changed due to the acetylation. The birefringence property of the cell walls at the lowest WPGs is very similar to the non-acetylated wood. Kumar & Agarwal (1983) reported that at weight gain of 13.5% in acetylated wood, about 86.4% of hydroxyl groups in lignin, 21.6% in hemicelluloses and 9.3% in cellulose are substituted. Polarized microscopy here confirms the former report in which crystalline cellulose was influenced due to the substitution of acetyl groups. Loss of birefringence property due to the acetylation (without attack) reveals that polarized microscope cannot be utilized for identification of decay patterns in acetylated wood.

A rapid invasion of tracheids and rays due to brown rot decay in the non-acetylated pine wood earlier than the beech wood indicates that the brown rot fungus has a preferential tendency to attack pine (as a softwood) than that the beech wood (as a hardwood). Whereas, the preference of the white rot fungus is to degrade the beech wood. The brown rot fungus attacked non-acetylated pine wood easier than beech wood. For example vessels and rays in beech were not severely attacked as tracheids and rays in pine wood (tables 4-2 & 4-3). The similar preference was followed in the acetylated wood. Whereas the white rot fungus attacked beech wood easier than pine wood. Fiber cell walls were severely eroded and removed by *T. versicolor* in beech wood, while tracheids could resist their integrities after severe attack. Similar preferential attack by white rot fungus was also distinguished in acetylated beech and pine. Same preferences in attacking acetylated soft and hardwoods were reported by some authors (Okino *et al.*, 1998; Suttie *et al.*, 1998). Schwarze *et al.* (2000) and Green & Highley (1997) reported that brown rot fungi attack softwoods more preferential than hardwoods and white rot fungi have more tendencies to attack hardwoods than softwoods. Ohkoshi *et al.* (1999), Takahashi *et al.*, (1989a&b), Takahashi (1996) and Okino *et al.* (1998) have also reported that brown rot fungi degrade acetylated softwoods faster than hardwoods and white rot fungi are more resistant to acetylated hardwoods than softwoods. A possible involvement of different chemical reactivities between the two types of lignin, namely the guaiacyl (softwood lignin) and the syringyl (hardwood lignin), and/or possible different cellular distributions of acetyl bond between softwood and hardwood are suggested. Investigations have shown that syringyl lignin degrades more rapidly than that guaiacyl lignin (Zabel & Morrell, 1992).

Microscopical studies of white- and brown-rot decayed wood showed that the acetylation at low weight gains (3.41% and 2.71% in beech and pine respectively) gives no protection to acetylated wood against both fungi and they could attack both wood species as severely as the non-acetylated one. Using the moderate acetylation process to achieve weight gains under about 10% in both species gives considerable protection to wood when it is compared to the low acetylation levels. Acetylation of wood above 10% of weight gains demonstrated reduction and inhibition in wood decay. In order to prevent decay, acetylation levels higher than the achieved weight gains are required.

6.4. Fungal protection mechanism and bioassays

Microscopical studies on soil bed samples showed that soft rot fungal hyphae colonized in the acetylated wood. The number of hyphae was less than the non-acetylated wood at raising weight gains. In addition to, ergosterol assay and microcalorimetry were used to monitor fungal biomass in the acetylated

wood and reveal microbial activities in soil exposed wood samples. Ergosterol content was increased at early stages of incubation which indicated a rapid colonization by hyphae. Ergosterol assays also showed decreasing amounts in the acetylated wood at increased weight gains and it was not detectable at the highest weight gains. Ergosterol assays also showed a higher degree of colonization in the non-acetylated wood and revealed a decrease in fungal biomass with increasing weight gains. Microcalorimetry confirmed the ergosterol results and showed a lower production of heat and energy at increasing degree of the acetylation.

Ergosterol assay and fluorescein diacetate (FDA) hydrolysis also showed that acetylation influenced colonization of white rot fungus *Trametes versicolor* and its biomass decreased at increased weight gains. Main colonization was determined in the non-acetylated wood and slight amount of colonization at the highest weight gain.

Hydrolysis of FDA showed that esterase activity was reduced due to the acetylation. No reaction with FDA at the highest weight gain (17%) revealed that the fungus had no metabolic activity in the highly acetylated wood. Whereas increasing FDA reaction in the non-acetylated wood and the lowest weight gain (2%) indicates that fungal active mass was increased in those woods. However, FDA hydrolysis was low in moderately acetylated wood comparing the non-acetylated one and the lowest weight gain. Overall FDA reaction increased during the incubation period, except at the highest weight gains. Variation of FDA reaction is very similar to ergosterol measurement. Represented results here indicate that both methods (ergosterol assay and FDA hydrolysis) monitor variation of fungal mass in all types of wood.

Microcalorimetry revealed that the bioactivity of soil microorganisms in wood decreased with increasing degree of the acetylation. The same tendency was confirmed by ergosterol measurements. Ergosterol measurements additionally revealed that fungi also contributed to the colonization of wood mainly during the early period of exposure. However, their mass decreased due to lack of nutrients especially when cell wall degradation was prevented by acetylation. Comparison between beech and pine wood revealed that microorganisms produce less heat in pine than in beech. It could be concluded that microorganisms degrade pine wood limitedly due to its higher bioresistance against the microorganisms. It has also been reported that beech is more susceptible to fungal decay than pine wood in the same conditions due to its different chemical and anatomical structure (Butcher & Nilsson, 1982).

White rot fungal activity in the acetylated wood was also monitored by using microcalorimetry in samples of solid state-fermentation test. Heat and energy production were influenced by the acetylation. They were decreased at raising weight gains. Microcalorimetry showed that fungi produce more amounts of heat and energy in the non-acetylated beech wood than that of the highest weight gain.

First raise of ergosterol content in soil bed samples indicates rapid colonization of fungi in wood, when fungi assimilate organic compounds from the soil. After consumption of the major amount of nutrients from the soil, the amount of ergosterol decreased due to lack of nutrients. In this phase, fungi metabolized their reserved ergosterol in mycelial walls (Braun-Lüllemann, 1989). Anatomical structure of wood such as open vessel lumina and readily available nutrients in ray cells may have contributed to the early and rapid colonization of the fungi through the vessels, ray cells and axial parenchyma in wood. Fungal succession could be considered as another reason for varying ergosterol amount during exposing to the soil. Käärik (1975) reported successional changes in the colonizing microorganisms and also successive changes in the attacking decay fungi.

Similar to our results, fungal colonization in wood was reported by other authors. Encinas & Daniel (1999) reported an early and rapid colonization and then reduction of a blue stain fungus *Lasiodiplodia theobromae* in birch and pine based on the measurements of ergosterol. The same was also reported for *Ceriporiopsis subvermispora* (Messner *et al.*, 1998).

Regarding the above explanation, the colonization phase occurs in the non-acetylated and the acetylated wood. However, fungal biomass decreases with raising the weight gains and was not detectable at the highest weight gain. Due to the inability to degrade the acetylated cell wall, fungi do not find nutrients in wood and probably metabolized deposited ergosterol in mycelial walls.

As microscopy revealed the presence of fungal mycelia in highly acetylated wood and ergosterol determination also showed no ergosterol content in these woods, the reason could be related to the ergosterol content in these hyphae which was under the detection limit or the hyphae did not contain

ergosterol due to metabolic degradation and oxidation upon cell death (Gessner & Schmitt, 1996) and the number of hyphae containing the ergosterol was less than detection limit.

In samples of solid-state fermentation, decreasing ergosterol content in the acetylated wood in comparison with the non-acetylated one indicated less fungal biomass in wood. On the other hands, microcalorimetry revealed less metabolic heat and energy at the highest weight gain due to soft rot and white rot fungal activity. While during the first weeks of incubation, the metabolic heat was higher than the end of incubation period. The metabolism was generally lower than the non-acetylated wood and also low weight gains. Heat production was compared between activities in samples of 14 and 84 days of incubation and indicated that the fungus was active during the first period of incubation in all acetylated wood samples and it was decreased at the end of incubation. However, it was increased in the non-acetylated beech wood. The explanation of this phenomenon could be in-/low-accessibility of cell wall polymers for fungal mycelia in the acetylated wood at low and moderate degrees of the acetylation, while they could be blocked for enzymatic activities due to high degree of the acetylation at WPG 17%. High heat production in the non-acetylated wood is related to higher activity of white rot fungus due to accessibility of cell wall polymers for hydrolyzing enzymes. Similar phenomenon was observed for energy production in all types of wood even in the non-acetylated one. It indicates that the fungus was active during the first days of the incubation and the activity was decreased to the end of the incubation period. Accumulative energy production showed that white rot fungus lose its activity with increased weight gains. Active fungal metabolism produces higher amount of heat in the non-acetylated wood, while it was less in the acetylated one. Insignificant fungal activity at the highest weight gain indicates that white rot fungus has ceased its bioactivity or might be dead due to blocked cell wall polymers and starvation in the acetylated wood. Consequently less heat was produced due to the less fungal mass in highly acetylated wood. Heat and energy consumptions were revealed also in highly acetylated wood. Probably, it was occurred due to improper sealing of testing vials during microcalorimetry. Therefore, some portion of heat might be escaped from closed system.

6.5. Mode of protection in acetylated wood

Earlier authors believed that bioresistance mechanism of the acetylated wood is related to changing the hydrophilic nature of wood and reducing moisture contents in cell walls due to substitution of hydroxyl groups with acetyl groups (Stamm & Baechler, 1960; Rowell, 1983). Foster *et al.* (1997) and Foster (1998) tested different chain anhydrides and reported that the bioresistance of acetylated wood is related to "bulking effect" of the chemical reagents during wood modification. Bulking of cell walls blocks small pores or reduces their sizes to smaller than the required sizes for enzyme penetration or blocking of cell wall micro-capillaries prevents the access of low molecular weight degradative agents produced by the fungus (Papadopoulos & Hill, 2002; Hill, 2002). Another hypothesis has related the protection mechanism of the acetylation to "chemical alteration" in cell wall polymers with blocking the active sites and making them inaccessible to fungal enzymes (Takahashi *et al.*, 1989a&b; Takahashi, 1996).

After sampling, acetylated wood samples were observed wet enough to provide the optimal moisture for fungal decay condition. Fungal mycelia contain conductive mycelia, which transfer water to dry areas to provide optimal moisture for hydrolyzing enzymes. Therefore, at least S_3 layer in pine tracheids and vessels in beech or luminal sides of other cells, which were in direct contact with hyphae, should have enough moisture to be attacked by fungi. Concerning the pattern of white rot decay in the non-acetylated wood, the white rot fungus thinned cell walls from lumina inward the ML. Therefore, luminal sides of the acetylated cells were wet enough to be attacked by the fungus. Whereas neither tracheid cells were not severely attacked, nor vessels were remained protected at the highest weight gains. The origin of the moisture in the samples is probably due to the breakdown of carbohydrates in media or slight breakdown of the cell wall polymers in attacked or adjacent samples (Suttie *et al.*, 1997&1998). Therefore the hypothesis of hydrophobisation could not be confirmed alone. On the other hands, slight brown rot attack and bore holes on the highly acetylated cell walls do not entirely confirm the hypothesis of "bulking effect" or "blocking of micro-capillaries" and reduction of small pores in the cell walls. Brown rot fungi use non-enzymatic system to attack cell walls by producing free radicals, which are produced from Fenton reaction and their sizes are smaller than the smallest pore sizes (< 3.8 nm) in wood cell walls (Flournoy *et al*, 1991; Highley *et al.*, 1994). Therefore, these diffusible agents can easily penetrate into the cell walls. It is reported that the smallest enzymes of brown rot fungi are too large to penetrate through the cell wall pores (Highley *et al.*, 1994). An average size for cellulases is about 5nm in diameter if the molecule is assumed to be spherical in shape or 3 by 17nm if ellipsoid. The size for lignin peroxidases is 4.7nm if their shape is considered to be

spherical and 4.3 by 6nm if the shape is considered to be ellipsoid. Manganase peroxidases have similar sizes as lignin peroxidases. Therefore, the evidences indicate that these enzymes cannot penetrate into cell wall pores, which have 2nm sizes (Flournoy *et al.*, 1993). However, small cellulases (1.5 to 4nm) and ellipsoid cellulases may thus gain access to cell walls at early stages if white rot decays wood and small molecule radical can diffuse into cell walls if brown rot fungus decays wood (Flournoy *et al.*, 1993). Reports indicate that oxalate support non-enzymatic decay system in wood cell walls at initial stage of decay by supporting the chemical reactions which provide some free radicals (Green *et al.*, 1998; Koenig, 1974; Highley *et al.*, 1994). Production of oxalate crystals in highly acetylated tracheid cell lumina indicates non-enzymatic activity of brown rot fungus. Brown rot fungi have the capacity to produce extracellular oxidative metabolites when the organisms are maintained under specific conditions, especially nitrogen and carbohydrate limitations (Highley *et al.*, 1994).

Consequently, the capability of the brown rot fungus to attack acetylated wood at higher weight gains in comparison with white rot fungus could be related to its non-enzymatic system. This fungus can attack acetylated wood by producing free radicals, which are smaller than enzyme. Concerning the mode of action in white rot decay, the presence of hydroxyl groups in cell walls is very important in initializing the attack by hydrolyzing enzymes (Eriksson *et al.*, 1990; Highley & Dashek, 1998). More acetyl substitutions in lignin, in comparison with cellulose, probably limit its ligninolytic activities, whereas brown rot fungus has more cellulolytic capability than ligninolytic (Eriksson *et al.*, 1990; Srebotnik & Messner, 1991). Therefore, less acetyl substitutions in carbohydrates (especially cellulose) permit brown rot fungus to attack acetylated wood even at higher weights gains. Rosenqvist (2001) showed by using labeled ^{14}C and tritium acetic anhydride that uniform distribution were detected under scanning electron microscope at higher weight gains (above 20%). Also Kumar & Agarwal (1983) reported uneven substitutions in cell wall components during the acetylation. Their report showed that acetylation of lignin is faster than hemicellulose and holocellulose (lignin>hemicelluloses>holocellulose) during the acetylation and at same weight gains substitution of hydroxyl groups in lignin is higher than hemicellulose and cellulose (*e.g.* at 13.5% of weight gain, 86.4% of hydroxyl groups in lignin, 21.6% in hemicelluloses and 9.3% in cellulose are substituted. In another report, Rowell *et al.* (1994) showed that at 18% of weight gain, almost all lignin hydroxyl groups were substituted, but only about 20% of the holocellulose hydroxyls were substituted. Therefore, Kumar & Agarwal (1983) suggested that higher lignin substitution indicates that acetylated wood should be more resistant to white rot fungi than brown rot.

Due to the decreased number of hydroxyl groups and smaller pore sizes at higher weight gains, cellulolytic and ligninolytic enzymes of white rot fungus can probably not diffuse into cell walls and fail to attack at higher weight gains. However the observed slight brown rot decays at higher weight gains and the scarcely produced bore holes on the highly acetylated cell walls puts theories of bulking effects and blocking of micro-capillaries under question. Therefore, it is probably suggested that whole cell walls were not uniformly acetylated during the acetylation. There exist probably some small regions in the cell walls, which were not acetylated as high as others and fungi could search for such small regions with low levels of acetylation to attack (concerning the produced bore holes by the brown rot fungus). Finally we can suggest complexity of factors that involve in protecting the acetylated wood against fungi such as lowered moisture content in cell walls that influences hydrolyzing enzymes, blocking of active sites in cell wall polymers that can influence some radical reactions, bulking effect and blocking of micro-capillaries (micro-pores) in cell walls that inhibit diffusion of small degrading agents and small regions in cell walls that gained lower degrees of acetylation in comparison with other close regions. Efficient level of the acetylation at above 10% of weight gain on white rot decay, probably relates to bulking effect of the cell walls and more limitation of enzymes to penetrate into the cell wall. While brown rot fungi use non-enzymatic system to degrade cell walls. Therefore degrading agent can penetrate and attack the cell walls slightly at least at higher weight gain. Anyhow, results showed that 10% of weight gains was efficient level to prevent cell walls.

6.6. Conclusion

Overall, the presented results have demonstrated that the acetylation of beech and Scots pine wood improves bioresistance of wood to against soil microorganisms and wood destroying white- and brown-rot fungi. Concerning the microscopical results and previous reports, a moderate acetylation (around 10%) could be an efficient level to inhibit fungal attack in wood and improve durability of wood species to higher

durability classes (such as II-III). However, full protection of wood could be achieved at above 20% of weight gains.

Microscopical studies showed that fungi colonize acetylated wood cell lumina, whereas no or slight decay occurs at higher weight gains. Study on the advancement of decay in acetylated wood showed that fungi cannot develop their decay to advanced stages and limit themselves to early or initial stages. However there are no differences between their decay patterns in acetylated and non-acetylated wood. Colonization of hyphae in cell lumina and ray cells indicate non-toxicity of acetylation to fungi; whereas it provides prevention against fungi.

Ergosterol assay and microcalorimetry are two methods in monitoring microbial activity and biomass in acetylated wood. Increased degree of the acetylation correlates with reduced ergosterol content in wood and lower heat production. The ergosterol content revealed that the amount of the fungal colonization correlates with the degree of acetylation.

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