Chemical and Ultrastructural Aspects of Thermally Modified Wood with Emphasis on Durability

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Abstract

The Termovuoto (thermo-vacuum) process is an environmental friendly industrial approach to modify wood by combining efficient vacuum drying and thermo-treatment. In this thesis, chemical and ultrastructural aspects of two softwoods (spruce and fir for 4 h at 160−220℃) and two hardwoods (ash and beech for 3 h at 190−220℃) thermally modified using the Termovuoto process were studied by light- and electron microscopy with emphasis on durability.

Histochemical staining indicated an increasing amount of acidic groups in thermally modified woods (TMWs), particularly in the compound middle lamella (CML) including middle lamella cell corner (MLcc) (CMLcc) regions of TMWs treated at 220℃ ($TMW_{220℃}$). TEM observations showed significantly increased $KMnO_4$ staining intensity of lignin in $TMW_{220℃}$, presence of electron dense particles in CMLcc regions of softwood $TMW_{220℃}$, and large lignin aggregates and disordered lamellar structure in the fibre S_2 layer of hardwood $TMW_{220℃}$.

The durability of TMWs against two white rot (*Phlebia radiata*, *Pycnoporous sanguineus*)-, two brown rot (*Postia placenta*, *Gloeophyllum trabeum*)- and three soft rot (*Chaetomium globosum*, *Phialophora mutabilis*, *Phialophora malorum*) fungi was evaluated by the soil block test. For brown- and white-rot fungi, Termovuoto treatment showed considerable improvement in durability class (i.e. class 1−3) for soft- and hardwoods at 220℃ against all fungal species tested. Softwood TMWs showed an overall lower decay resistance than hardwood TMWs, among which ash TMWs showed greater durability than beech TMWs. For soft rot fungi, softwood TMWs were more durable than hardwood TMWs, irrespective of fungal species. Ash showed lower durability than beech in untreated reference wood, while ash TMWs showed greater durability than beech TMWs during one year decay test. Behavior of thermal modification (TM) differed significantly between ash (ring-porous hardwood) and beech (diffuse-porous hardwood) against brown-, white- and soft-rot fungi, indicating importance of the native wood anatomy.

Decay patterns and morphological changes of TMWs were examined by light- and electron microscopy. The white rot fungus *P. sanguineus* did not show significant differences in characteristic features of decay in tracheids and fibres of TMWs compared to those in untreated reference. However, the delignification process in tracheids and fibres by *P. sanguineus* was delayed in TMWs, particularly at high treatment temperatures as evidenced by narrower transition zones from delignified and lignified areas than untreated reference. The soft rot fungus *P. mutabilis* produced typical soft rot Type-I cavities in fibres of hardwood TMWs at low temperature (190−200℃). However, soft rot cavity formation was greatly inhibited and/or delayed

in fibres at high treatment temperatures (i.e. 210−220℃). Ash $TMW_{200℃}$ showed a radial-like distribution of electron dense materials in cavities and lack of fibrillar-like materials within degraded fibre walls, which differed from reference.

The fungal durability of Termovuoto TMWs differed in terms of treatments, wood and fungal species. The Termovuoto process did not change the patterns of decay caused by white-, brown- and soft-rot fungi used, but rather slowed down the decay process at certain treatment temperatures for certain wood species. Understanding of the decay patterns in TMWs is essential for further optimization of the Termovuoto process for improving the durability of specific wood species for specific purposes.

Keywords: brown rot, decay resistance, light microscopy (LM), scanning electron microscopy (SEM), soft rot, thermo-vacuum (Termovuoto) process, thermally modified wood (TMW), transmission electron microscopy (TEM), ultrastructure, white rot.

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Dedication

To my family…

Contents

List of Publications

This thesis is based on work contained in the following papers:

I **Gao, J.**, Kim, J.S., Terziev, N., Allegretti, O. and Daniel, G. 2014. Chemical and ultrastructural changes in compound middle lamella (CML) regions of softwoods thermally modified by the Termovuoto process. *Holzforschung* 68 (7): 849−859.

II Kim, J.S., **Gao, J.**, Terziev, N., Cuccui, I. and Daniel, G. 2015. Chemical and ultrastructural changes of ash wood thermally modified using the thermo-vacuum process: I. Histo/cytochemical studies on changes in the structure and lignin chemistry. *Holzforschung* 69 (5): 603−613.

III **Gao, J.**, Kim, J.S., Terziev, N. and Daniel, G. 2016. Decay resistance of softwoods and hardwoods thermally modified by the Termovouto type thermo-vacuum process to brown rot and white rot fungi. *Holzforschung* 70 (9): 877−884.

IV **Gao, J.**, Kim, J.S., Terziev, N. and Daniel, G. 2017. Effect of thermal modification on the fungal durability and decay patterns of hardwoods and softwoods against soft rot fungi. *International Biodeterioration & Biodegradation.* (In press)

V **Gao, J.**, Kim, J.S. and Daniel, G. 2017. Effect of thermal modification on the micromorphology of decay of hardwoods and softwoods by the white rot fungus *Pycnoporus sanguineus* (*Holzforschung*; Submitted).

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The contribution of Jie Gao to the papers included in this thesis was as follows:

I J.G. participated in the design of the experiments, carried out practical and analytical work, and wrote the first draft of the manuscript.

II J.G. participated in the design of the experiments, carried out practical and analytical work, and had responsibility for drafting the manuscript.

III J.G. participated in the design of the experiments, carried out practical and analytical work, and had responsibility for drafting the manuscript.

IV J.G. designed experiments, carried out practical and analytical work and had the main responsibility for article writing.

V J.G. designed experiments, carried out practical and analytical work and had the main responsibility for writing the article.

Additional papers included in the thesis but not discussed in the thesis summary:

VI Kim, J.S., **Gao, J.**, Terziev, N., Allegretti, O. and Daniel, G. 2015. Chemical and ultrastructural changes of ash wood thermally modified (TMW) using the thermo-vacuum process: II. Immunocytochemical study of the distribution of noncellulosic polysaccharides. *Holzforschung* 69 (5): 615−625.

VII Kim, J.S., **Gao, J.** and Daniel, G. 2015. Ultrastructure and immunocytochemistry of degradation in spruce and ash sapwood by the brown rot fungus *Postia placenta*: Characterization of incipient stages of decay and variation in decay process. *International Biodeterioration & Biodegradation* 103: 161−178.

VIII Kim, J.S., **Gao, J.** and Daniel, G. 2015. Cytochemical and immunocytochemical characterization of wood decayed by the white rot fungus *Pycnoporus sanguineus* I. preferential lignin degradation prior to hemicelluloses in Norway spruce wood. *International Biodeterioration & Biodegradation* 105: 30−40.

IX Kim, J.S., **Gao, J.** and Daniel, G. 2015. Cytochemical and immunocytochemical characterization of wood decayed by the white rot fungus *Pycnoporus sanguineus* II. Degradation of lignin and non-cellulosic polysaccharides in European ash wood. *International Biodeterioration & Biodegradation* 105: 41−50.

Abbreviations

CML	Compound middle lamella
CMLcc	Compound middle lamella including middle lamella cell corner
G-lignin	Guaiacyl-lignin
LM	Light microscopy
MC	Moisture content
MLcc	Middle lamella cell corner
S-lignin	Syringyl-lignin
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TM	Thermal modification
TMW	Thermally modified wood

1 Introduction

1.1 Wood modification

Wood is one of the world's most excellent and abundant natural materials, which is biologically degradable, environmentally friendly and renewable, and has reasonably good mechanical properties. It has been widely used for millennia and is still indispensable for fulfilling varying purposes even today. For example, wood can serve as construction materials and can be used for furniture manufacturing and toy-making, not to mention important energy contributions coming from wood-derived fuels. Despite its uses and advantages, natural wood materials suffer from drawbacks that arise from their durability and dimensional stability. Many wood species do not give adequate performance and have limited durability against ageing and biodegradation under certain conditions.

Over past decades, considerable achievement in wood modification has advanced techniques of chemical and physical treatments, so as to improve the properties of wood material for *in-service* use (Hill, 2011). The aims of wood modification are not only to improve the important properties of wood, such as dimensional stability, stiffness and hardness, but also to enhance the resistance of wood against biodegradation from fungi, bacteria, termites and insects, and thereby extend the service life of wood in use (Homan & Jorissen, 2004).

With increasing public awareness of environmental problems, the demand for novel methods to produce sustainable and non-toxic wood products without application of toxic chemicals has also been steadily increasing. Among the investigated approaches [e.g. thermal treatments, furfurylation, acetylation, hydroboration with silicon treatments, modification with 1,3-dimethylol-4,5-dihydroxy ethylene urea (DMDHEU), treatment with oil/wax/paraffins] that have been known for a long time particularly in European countries (Finland, Netherlands, France, Austria, Germany, Russia, etc.), thermal treatments have

drawn a great deal of attention for producing economically, ecologically and environmentally friendly wood products (Sandberg *et al.*, 2013; De Vetter *et al.*, 2010; Dieste *et al.*, 2009; Homan & Jorissen, 2004).

1.2 Thermal modification

A variety of thermal modification (TM) technologies have been introduced over the decades, and include ThermoWood in Finland, Plato process in Netherlands, oil heat treatment (OHT-Process) in Germany, and the Les Bois Perdure and Retification processes in France (Esteves & Pereira, 2009; Homan & Jorissen, 2004). The sales of modified wood materials and products have thus increased greatly over the last few years (Production statistics, 2014). While such modification methods differ from each other, in terms of shield gas (nitrogen or steam), protecting liquid used and humidity requirements of wood (wet or dry) (Xie *et al.*, 2002; Rapp, 2001), they all have one thing in common: they modify the chemical structure of wood at temperatures ranging from 160 to 260℃.

For selected wood species, different TM approaches can be applied with the performance gain often dependent on treatment level and wood species used. For example, TM at elevated temperatures can lead to permanent chemical modification of the cell wall (i.e. changes in hemicelluloses, cellulose and lignin), thereby improving decay resistance, reduced moisture deformation and increased dimensional stability (Hill, 2011). At the same time, treatments can render the wood to distinct shades of darker colours for final products. The degree of changes in wood properties are dependent on the process type, the wood species to be treated, the maximum temperature reached in the process, and the duration at that temperature, etc. As a result, TM processes can yield a high quality product with many excellent properties and outlook for exterior and interior applications, such as wall cladding, siding, garden furniture, window frames, doors, musical instruments and cupboards. The production of thermally modified wood (TMW) has been progressively increasing since 2001. For example, ThermoWood global production grew nearly 8 fold, from 18,799 m^3 in 2001 to 145,733 m^3 in 2014 (Production statistics, 2014).

1.2.1 ThermoWood

ThermoWood was developed in the early 90's at the Finnish Research Centre (VTT) for both softwood and hardwood species and is the most widely applied industrial TM process in Europe, as reflected by the highest production rate. With this method, the wood is treated at temperatures above 180℃ under atmospheric pressure. According to the Finnish ThermoWood Handbook

(2003), the process is constituted of three main stages: 1) warming up, 2) drying, and 3) cooling and conditioning. During the warming-up process, the temperature rises quickly to around 100℃ and then increases steadily to 130℃ in order to heat and pre-dry the timber. A large amount of steam is generated at this stage, and the moisture content in the wood decreases to almost zero due to high temperature drying. During the drying phase, the temperature inside the kiln is increased between 185 and 215℃ depending on the application of the treated products and is then maintained at that level for a 2 to 3 h plateau. In the final stage, the temperature of the timber is reduced to 80−90℃ by using water spray systems, which is followed by conditioning that moistens the heat treated timber and reduces its moisture content to 4−7%.

The use of water steam replaces air and builds low oxygen conditions, so as to prevent the wood from burning and cracking at high treatment temperatures. However, the generation of vapour and the long pre-heating time in turn significantly increase the costs of the above process.

1.2.2 Oil heat treatments (OHT-process)

The OHT-process differs from most other heat treatments in that it is carried out using a hot-oil bath. Through application of hot oil, oxygen is excluded from the wood during treatment with the heat transferred to the wood reaching up to 180−260℃ during the process (Rapp & Sailer, 2000).

Wood samples with an initial moisture content of 12% are placed directly in a hot-oil bath without preheating. Extra processes for heating and cooling may be required if the dimension of wood samples are large. Although the oil bath ensures good heating and deficiency of oxygen during treatment, the oil (e.g. linseed oil) has an unpleasant smell and the wood can absorb a large amount of oil (ca. 50−70% mass increase) which are major disadvantages.

1.2.3 Plato

The Plato process (Militz & Tjeerdsma, 2001) modifies wood by combining hydrothermolysis with a dry-curing step. The hydrothermolysis process speeds up the reactivity of cell wall components resulting in chemical transformations. Plato consists of two steps, in which green or air-dried wood heated in water under high pressure (saturated steam conditions) is subjected to temperatures between 160 and 190℃. After the wood’s moisture content reaches 10%, a curing step is performed at atmospheric condition with temperatures in the range 170−190℃.

1.2.4 Retification and Les Bois Perdure

For the Retification process, pre-dried wood (ca. 12% moisture content) is slowly heated to 210−240℃ in a nitrogen atmosphere of less than 2% oxygen content. With the Les Bois Perdure process, fresh wood can also be treated. The wood samples pre-dried in an oven are heated to 230℃ under a steam atmosphere. Studies have shown that the higher the treatment temperature, the more durable the wood becomes, but the greater strength loss induced. Therefore it is essential to control accurately the temperature. However, both the above processes are very sensitive to slight changes in temperature, since the maximum temperature has a major impact on durability and mechanical properties. (Vernois, 2001)

1.3 Termovuoto

The Termovuoto process is a new industrial TM methodology that has been developed by the National Research Council of Italy - Tree and Timber Institute (CNR-IVALSA), and is based on the combination of an efficient vacuum drying process with thermal treatment process (Ferrari *et al.*, 2013; Allegretti *et al.*, 2012). It is actually a thermo-vacuum process. The wood is first dried in air at 100℃ until the moisture content reaches 0%. Thereafter, TM is performed in the same chamber by increasing the temperature to 160−220℃. A vacuum pump is used at this stage to remove the residual air and maintain the vacuum. Compared to other TM processes, Termovuoto is more promising and has several advantages, including lower energy and time consumption, easier and cheaper management of the volatile wastes, less corrosion and lower mass loss of wood and no odor development. These effects are probably due to the action of the vacuum pump that continuously removes volatile products that can cause accelerated degradation of polysaccharides in wood cell walls during processing (Ferrari *et al.*, 2013; Allegretti *et al.*, 2012). Several soft- and hardwood TMWs produced by the Termovuoto process have also been found to yield satisfactory mechanical properties and decay resistance.

1.4 Research on thermally modified wood (TMW)

1.4.1 Chemistry and physical properties

TMW is always accompanied by chemical degradation and/or modification of hemicelluloses, cellulose and lignin, which means the strength properties of the wood are modified because of changes in chemical structure of cell wall components. The changed wood composition results in lower hygroscopicity

and improved dimensional stability of the wood and increased resistance to fungal decay (Hill, 2006). However, high temperature and long process duration can lead to some undesired side effects, such as strength loss and increased brittleness for the treated wood, limiting its use as a commercial material (Salmén *et al.*, 2008; Boonstra *et al.*, 2007).

The chemical and physical properties of TMWs have been extensively investigated by using a set of methodologies, such as classical wet chemistry, Fourier transform infrared (FTIR)- and nuclear magnetic resonance (NMR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and ultraviolet microspectrophotometry (UMSP) (Dubey *et al.*, 2012; Esteves & Pereira, 2009; Windeisen *et al.*, 2007; Tjeerdsma & Militz, 2005; Sivonen *et al.*, 2002). Such studies have provided detailed information regarding changes in structure and chemical bonding resulting from thermal treatment, but have shed little light on changes in the chemistry of TMWs at tissue and cellular levels, e.g. the chemical and structural changes in a specific cell wall layer following thermal treatment. Previous microstructural studies on TMWs have also mostly concentrated on changes in anatomy, porosity and pore-size distribution in cell walls, crystallinity, and microfibril angles (MFAs), using light- and electron microscopy, differential scanning calorimetry and X-ray scattering/diffraction (Biziks *et al.*, 2013; Brandt *et al.*, 2010; Zollfrank & Fromm, 2009; Boonstra *et al.*, 2006a; Boonstra *et al.*, 2006b).

1.4.2 Resistance to biological degradation

Fungal attack is responsible for significant morphological changes in wood structure and dramatic alterations of the physical properties and chemical composition of wood materials with profound implications for dimensional stability (e.g. archaeological objects). Thermal modification improves the resistance of wood against biological degradation in a way that the treated wood can no longer serve as a readily available nutrient medium for the enzymes and catalysts of degrading fungi (i.e. at least initially) (Chaouch *et al.*, 2010). Thus, fungal durability is of key consideration to evaluate TMWs and the risks associated with use of TMW products *in-service*.

Under natural conditions and on the basis of physical and chemical changes produced and resulting alterations in color of decayed wood, wood decay fungi are primarily classified as brown-, white- and soft-rot.

Most previous studies concerning the fungal resistance of TMWs have focused on brown- and white-rot fungi (basidiomycetes) (e.g. Candelier *et al.*, 2012; Chaouch *et al.*, 2010; Šušteršic *et al.*, 2010; Hakkou *et al.*, 2006; Kamdem *et al.*, 2002), but little research has been made to evaluate the fungal resistance of TMWs against soft rot fungi. Unlike basidiomycetes, soft rot

commonly occurs in wood exposed to more extreme conditions (e.g. high moisture situations and wood treated with preservatives) that can hinder both colonization and attack by more aggressive basidiomycetes (Daniel, 2003; Daniel & Nilsson, 1998). This emphasizes the importance of extension of our understanding on durability of TMWs against soft rot fungi to evaluate the possibility of use of TMWs in outdoor out of ground situations where high moisture content can exist even if periodically.

The characteristic mode of decomposition of wood by fungi varies depending on fungal species as discussed below. Differences in the anatomy and chemistry between wood materials (e.g. softwoods, hardwoods) can also lead to variations in the mode of decay by the same fungal species. The TM of wood commonly induces changes in lignin chemistry as well as in the chemistry of polysaccharides and wood cell wall structure (Esteves & Pereira, 2009). This suggests that decay patterns in TMWs can differ from general decay patterns of fungi. However, the effect of TM on morphological decay patterns of brown-, white- and soft-rot fungi is almost unknown. Furthermore, information on fungal degradation of cell wall components in TMWs, in particular at the cellular level is still lacking.

Brown rot fungi decay

Brown rot caused by basidiomycete fungi is generally considered the most common and destructive rot type for wood *in-service*, particularly in temperate geographic areas. Brown rot is characterized by a selective and rapid depolymerization of the cellulose of wood cell walls, without causing substantial lignin loss. The underlying mechanisms of brown rot decay have been extensively studied over the last decade (e.g. Arantes & Goodell, 2014; Goodell, 2003). The hyphae of brown rot fungi commonly colonize the wood cell lumen and attach to the S_3 layer with the decay process proceeding preferentially in the S_2 layer with first signs of attack often recognized at the S_2–S_1 interface. Often no visible morphological changes are observed in the S_3 layer until very advanced stages of decay. Enzymes responsible for the degradation of cellulose are thought too large to penetrate unmodified cell walls. Thus a non-enzymatic process (i.e. via Fenton reaction), has been advocated at least in the early stages of degradation by generating hydroxyl radicals so as to decompose the long chains of cellulose into small fragments (Goodell, 2003; Daniel *et al.*, 2007; Jensen *et al.*, 2001).

White rot fungi decay

White rot basidiomycetes decompose lignin as well as cellulose and hemicelluloses in wood (Daniel, 2014). The capacity for efficient lignin degradation in wood ensures the potential of white rot fungi in the pulp and

paper area. In this regard, the enzymes (e.g. lignin-/Mn peroxidases, laccase) involved in the degradation of lignin have been extensively investigated (Ten Have & Teunissen, 2001; Tuor *et al.*, 1995; Daniel *et al.*, 1989). For example, the widely studied white rot fungus *Pycnoporus sanguineus* used in this study can synthesize laccases capable of enduring high temperature that are particularly interesting for bio-bleaching of pulp and bioconversion of lignocellulosic materials (Pointing *et al.*, 2000). The decay patterns of white rot fungi vary in the rates in which lignin is removed in relation to degradation of polysaccharides. In this respect, white rot fungi can also be classified on the basis of their decay patterns as causing "selective" or "simultaneous" white rot. Selective white rot preferentially degrades and removes lignin from wood cell walls with cellulose remaining intact although modified. By contrast, simultaneous white rot fungi degrade lignin along with all the cell wall polysaccharides causing homogeneous cell wall decay and erosion. However, degradation patterns can vary greatly depending on fungal species, strains, environmental and wood conditions (Eriksson *et al.*, 2012; Singh & Singh, 2014). Some fungi can cause selective and simultaneous decay in the same wood samples but at different locations, while some species can switch decay patterns between preferential and simultaneous degradation over time (Daniel, 2013). Even different strains of a single species can also show considerable variations in lignin degradation.

Soft rot fungi decay

Soft rot fungi can tolerate a wide range of temperatures, humidity and pH conditions and decay a variety of wood substrates (Daniel, 2014). In contrast to brown- and white-rot fungi, soft rot decay commonly occurs in wood exposed to high moisture conditions or wood treated with preservatives that are capable of hindering both colonization and attack by the more aggressive basidiomycetes. Micromorphologically, soft rot decay differs from that caused by either brown- or white-rot fungi in several aspects. Decay normally involves a T-branching and/or L-bending process, and generation of characteristic cavities (Type I attack), and/or the complete erosion of the secondary wall (Type II attack) leaving a relatively intact middle lamella region (Daniel, 2016). The major chemical changes in wood derived from soft rot are quite similar to that from brown rot fungi and involve intense degradation of cellulose and hemicelluloses. It is commonly assumed that the nature of lignin, including concentration, condensation and composition (i.e. guaiacyl and syringyl lignin) significantly affect soft rot degradation of wood cell walls, in particular the formation of soft rot cavities (Daniel, 2016).

1.5 Objectives

The aims of my work are to study the chemical changes, structural properties, decay resistance and decay patterns of Termovuoto process treated TMWs at the cellular level by employing light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) combined with histo/cytochemistry and immunocytochemistry. Specifically, I have concentrated on the following three aspects:

- Evaluation of the effect of Termovuoto process on the chemical and ultrastructural changes in softwoods (spruce and fir) and hardwoods (beech and ash) at the cellular level, with special emphasis on changes in lignin chemistry via histo/cytochemical studies;

- Examination of the decay resistance of soft- and hardwood TMWs against brown-, white- and soft-rot fungi. Involves determining the fungal durability of TMWs with respect to treatment parameters (i.e. temperatures) and fungal species applied;

- Ultrastructural and cytochemical characterization of soft- and hardwood TMWs degraded by white- and soft-rot fungi, with focus on changes in decay patterns following Termovuoto treatment.

2 Materials and methods

2.1 Wood materials

Norway spruce (*Picea abies* Karst.), silver fir (*Abies alba* Mill.), European ash (*Fraxinus excelsior* L.) and European beech (*Fagus sylvatica* L.) used throughout the study were obtained from the Val di Fiemme forest (at an average altitude of 1757 m), Trentino region, Italy. Thermal modification (TM) of boards was carried out by the Termovuoto (thermo-vacuum) process according to the scheme described by Ferrari *et al*. (2013) and Allegretti *et al*. (2012). Spruce and fir (softwoods) were treated at temperatures 160, 180, 200 and 220℃ for 4 h under 240−260 mbar. Ash and beech (hardwoods) were treated at temperatures of 190, 200, 210 and 220℃ for 3 h under 240−260 mbar.

2.2 Fungi

Two brown rot-, two white rot- and three soft-rot fungi were used in the studies namely:

- Brown rot fungi: *Postia placenta* (Fries) Cooke (QM1010), *Gloeophyllum trabeum* (Persson: Fries) Murrill (BAM Ebw 109);

- White rot fungi: *Pycnoporus sanguineus* (Linnaeus: Fries) Murrill, *Phlebia radiate* (Fries) (L12-41);

- Soft rot fungi: *Chaetomium globosum* (Kunze: Fries, Telemorph) (strain F-171-1, ATCC 34152) (syn=*Chaetomidium japonicum*), *Phialophora malorum* (M. N. Kidd & A. Beaumont) McColloch and

Phialophora mutabilis (J. F. H. Beyma) Schol-Schwarz (1970) (syn=*Lecythophora mutabilis*).

All fungi were obtained from the culture collection, maintained at the Department of Forest Products, Swedish University of Agricultural Sciences.

2.3 Durability testing of TMWs

Soil block decay test was conducted according to AWPA E10-08 (AWPA Technical Subcommittee, 2008) with some minor modifications in wood sample size and incubation time. Test blocks were cut from TMW boards (30×100×1000 mm), numbered and oven-dried overnight at 103 ± 2℃ to measure the initial dry weight. Untreated blocks served as reference. The fungi described above were re-cultured on 2.5% w/v malt extract agar (MEA) plates for two weeks. One plate of each fungus was homogenized with 100 mL deionized water for further inoculation as described below.

- For brown rot fungi:
  Glass culture jars (500 mL) were half-filled with moist commercial planting soil. Scots pine (*Pinus sylvestris* L.) feeder strips (8×20×62 mm) were soaked in water for 10 min before being placed on the soil surface in the jars. After sterilizing the jars (103 kPa at 121℃ for 30 min) the test blocks (5×20×20 mm) were horizontally placed on the surface of the feeder strips.

- For white- and soft-rot fungi:
  Erlenmeyer glass flasks (100 mL) were used and half-filled with moist commercial planting soil. Test blocks (for white rot fungi: 5×20×20 mm; for soft rot fungi test: 5×10×30 mm) were vertically placed in the soil of the flasks with 5 mm of their length protruding above the soil surface.

Subsequently, the jars and flasks were sterilized at 103 kPa and 121℃ for 30 min. After cooling, the jars and flasks were inoculated by 6 and 4 mL homogenized fungal solution for brown- and white/soft-rot fungi, respectively, and placed in a dark culture room at 25℃ and 75% relative humidity to promote fungal growth. For soft rot decay test, the jars and flasks were weighed monthly to control the moisture content (i.e. 70−75%) by adding sterilized deionized water during the whole test period. The moisture content of the test wood blocks was also monitored during the whole decay process to

validate the test and for further analysis. The decayed blocks were removed at regular time intervals for mass loss (%) determination and microscopy observations.

2.4 Sample preparation for microscopy

2.4.1 Sample embedding

Small pieces (ca. 1×1×3 mm) were removed from untreated reference and TMW blocks with a razor blade. After fixation with a mixture of 2.5% v/v glutaraldehyde (GA) and 2% v/v paraformaldehyde (PA) in 0.05−0.1 M sodium cacodylate buffer (pH 7.4) for 3−4 h at room temperature, samples were dehydrated in a graded ethanol series (20−100%) and embedded in London Resin White (LR White, Basingstoke, UK) according to Kim & Daniel (2012). Some small sectors were also collected from fungal degraded wood blocks and embedded in LR White resin according to the procedures described above. For observations of soft rot decayed wood blocks, small pieces removed from the sectors were post-fixed in 2% w/v osmium tetroxide in 0.1 M sodium cacodylate buffer for 3 h at room temperature after fixation with a mixture of GA+PA.

2.4.2 Light microscopy

Small blocks (ca. 1×1×1 cm) removed from untreated reference and TMW blocks were immersed in distilled water overnight. Thereafter, transverse sections (ca. 10−30 μm) were prepared with a Leitz sliding microtome (Wetzlar, Germany). Some sections were also made from entire degraded wood blocks to provide information on the colonization process of fungi from the outside to the inside of wood blocks. After staining with 1% w/v toluidine blue in 1% borax (pH 8.5) or 1% w/v safranin O, sections were mounted in 50% v/v glycerol or with Canada balsam mounting medium. Sections were examined using a Leica DMLB light microscope (Wetzlar, Germany) equipped with an Infinity X-32 digital camera (DeltaPix, Samourn, Denmark). Semi-thin resin sections (ca. 1−2 μm) prepared from LR white embedded wood blocks using a Leitz rotary microtome (Wetzlar, Germany) or a Reichert ultra-microtome (Wien, Austria) were also examined according to the procedures described above. To visualize lignin distribution, thin sections (ca. 20−40 μm) were stained according to Wiesner and Mäule reactions (Nakagawa et al. 2012).

2.4.3 Transmission electron microscopy (TEM)

Transverse ultrathin sections (ca. 90 nm) were cut on a Reichert ultra-microtome (Wien, Austria) and examined by a Philips CM12 TEM (Eindhoven, Netherlands) at 80 kV for further analysis. Negative TEM films were digitalized by a film scanner (Epson Perfection Pro 750, USA). To detect lignin, ultrathin sections were stained with 1% w/v $KMnO_4$ in 0.1% w/v sodium citrate. For further details see Papers I and II.

2.4.4 Scanning electron microscopy (SEM)

Small blocks (ca. 3×5×5 mm) removed from *P. sanguineus* degraded blocks were fixed and osmicated as described above. After subsequent washing in 3×sodium cacodylate buffer (20 min each), samples were dehydrated in a graded ethanol series (20−100%) followed by an acetone/ethanol series (1:3, 1:1, 3:1 and 100% acetone), with three transfers made in absolute acetone. Samples (submerged in pure acetone) were subsequently dried in a AGAR CPD critical point drying apparatus (Agar Scientific Ltd., England) using CO_2 as the transition fluid. After drying, samples were mounted on aluminum stubs, coated with a layer of gold using an Emitech K550X sputter coater (Ashford, England), and finally examined and photographed under a Philips XL30 (Eindhoven, Netherlands) scanning electron microscope using an accelerating voltage of 10−15 kV.

3 Chemical and ultrastructural changes of Termovuoto treated softwoods and hardwoods

3.1 Histochemical observations of softwood TMWs

The color changes of spruce TMWs (i.e. thermally modified woods) are shown in Figure 1. The blocks of TMWs show a gradual decrease in yellowness and increasing brownness with increasing treatment temperature from 160 to 220℃. The color of unstained transverse thin sections changed from white to orange/reddish through the treatment process (Figure 1a−e). A similar trend was observed in the change of color for fir TMWs. Compound middle lamella (CML) including middle lamella cell corner (MLcc) (CMLcc) regions show an overall stronger orange/reddish color than secondary cell walls of tracheids (Figure 1f). By increasing treatment temperatures up to 220℃, the toluidine blue staining color was shifted from blue to greenish for tracheid secondary cell walls (Figure 1h−l), and from dark blue to light orange/yellowish for CMLcc regions (arrows in Figure 1g).

Toluidine blue is a polychromatic dye with high affinity for acidic polymers and reacts with different tissue components generating characteristic colors (Sridharan & Shankar, 2012; O'brien *et al.*, 1964), i.e. the different coloration of toluidine blue staining is positively correlated to the number of acidic groups present in tissues. Thus, changes in the staining color in tracheid cell walls (i.e. blue to green) and CMLcc regions (i.e. dark blue to orange) following thermal treatment reflect an increase of acidic groups in TMWs, particularly in CMLcc regions, which is presumably due to new carboxylic- and acidic phenolic groups generated by degradation or chemical modification of lignin and non-cellulosic components (Esteves & Pereira, 2009; Windeisen *et al.*, 2007; Sivonen *et al.*, 2002; Tjeerdsma *et al.*, 1998).

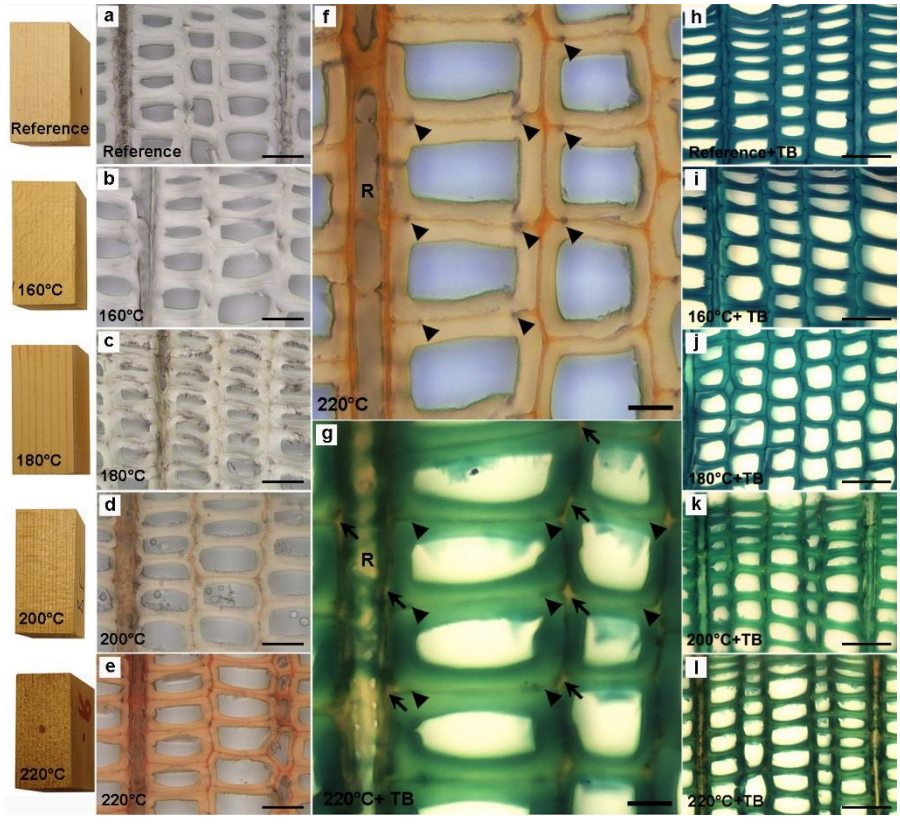

Figure 1. Changes in native wood/cell color and structure in spruce TMWs. The color of thin sections shifted from white in reference (a) to orange/reddish in TMWs (b−e), with stronger coloration in CMLcc regions than secondary cell walls (f). Note the formation of small cracks in latewood tracheids of $TMW_{220°C}$ (arrowheads in f, g). After staining with toluidine blue (TB), the color shifted from blue in reference (h) to greenish in TMWs (g, i−l) for the tracheid secondary cell walls, and from dark blue (h) to light orange/yellowish (arrows in g, i−l) for the CMLcc regions. Scale bars = 30 µm (a−e, j−i), 10 µm (f, g).

Small cracks were often detected between latewood tracheids in TMWs treated over 200℃ (arrowheads in Figure 1f, g). Interestingly, Bernabei & Salvatici (2016) found the explosion of bubbles in similar regions of spruce latewood tracheids during heat treatment using *in-situ* environmental scanning electron microscopy (ESEM) and hypothesized that the bubbles indicate the sudden release of steam and other gases from the cell walls. Presumably, this appears to be a common feature of TMWs and reflects dimensional changes in the cells (possibly swelling and contraction of the S_1 layer) during the treatment process.

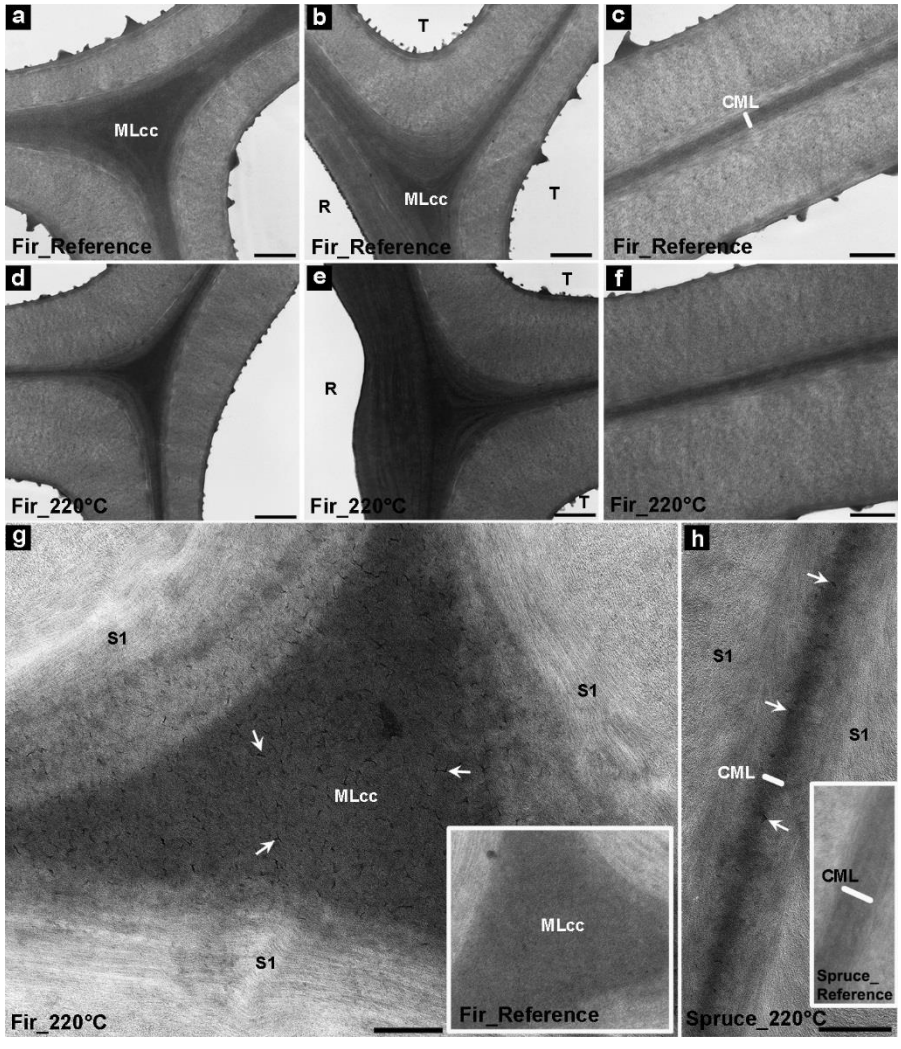

Figure 2. Changes in ultrastructure and lignin distribution in fir and spruce $TMW_{220°C}$. Stained with $KMnO_4$. The intensity of $KMnO_4$ staining in tracheids (T) and ray parenchyma cells (R) was stronger in $TMW_{220°C}$ (d−f) than reference (a−c). Note the formation of electron dense particles in CMLcc regions of tracheids of $TMW_{220°C}$ (arrows in g, h). These particles were not detectable in reference (insets in g, h). Scale bars = 1 µm (a, b, d, e), 500 nm (c, f−h).

After staining with $KMnO_4$, ultrathin sections from both reference and $TMW_{220°C}$ were examined by TEM (Figure 2). The differences in both lignin concentration and distribution in CMLcc regions between reference and TMWs were compared at the ultrastructure level. Fir and spruce TMWs showed almost identical staining features in the xylem cells. The staining intensity in secondary cell walls and CMLcc regions of tracheids and ray cells was

significantly increased by the treatment (Figure 2a−f), consistent with our light microscopy observations (Figure 1; Paper I) and previous investigations in which an apparent and relative increase in lignin content of TMWs was reported (Esteves & Pereira, 2009; Windeisen & Wegener, 2008; Boonstra & Tjeerdsma, 2006). It is generally considered that the apparent increase of lignin content in TMWs is due to the increased ratio of lignin by degradation of other cell wall components or by the polycondensation reactions of lignin with other cell wall components, rather than by generation of new lignin during treatment (Esteves & Pereira 2009). Degradation of polysaccharides and formation of abundant electron dense particles in CMLcc regions of tracheids and ray cells following thermal treatment may particularly reflect this aspect (Figure 2g, h; Paper I). Destruction of highly lignified warts in tracheids was also frequently detected in fir TMWs, as shown by both reduction in size and loss (Figure 2a−f).

3.2 Histochemical observations of hardwood TMWs

The change in color of ash TMWs was overall similar to that of softwoods (spruce, fir) with increase of treatment temperature, i.e. a gradual decrease in yellowness and increasing brownness in wood blocks and change from white to orange/reddish in transverse thin sections (Figure 3a, c, d). Similar changes in the color of toluidine blue staining as softwood TMWs were also detected in hardwood TMWs, indicating an increase of acidic groups by thermal treatment (Figure 3b, e, f). Like tracheids of softwood TMWs, hardwood TMWs also showed formation of small cracks between latewood fibres above 200℃ (arrowheads in Figure 3d).

To understand the relationship between lignin distribution in ash reference wood and variations in color changes between cell types in ash TMWs, lignin distribution in ash reference wood were examined using Mäule and Wiesner reactions (Figure 4). The Mäule reaction stains red and yellow for syringyl (S) and guaiacyl (G) lignin, respectively and Wiesner reaction for total lignin detection (i.e. pinkish-red staining color). For ash reference, the vessels showed a weaker Mäule reaction (Figure 4a, b) in early- than latewood but revealed the opposite patterns for Wiesner reaction (Figure 4e, f). The axial- and ray parenchyma cells revealed stronger Mäule and Wiesner reactions than fibres (Figure 4c, g). Wiesner reaction in the CMLcc regions of fibres was stronger than that in secondary cell walls (Figure 4e−g). These variations generated in ash reference suggest that the distribution and concentration of S/G lignin and total lignin content differ greatly depending on early-/latewood, cells types, cell wall regions and size of fibres.

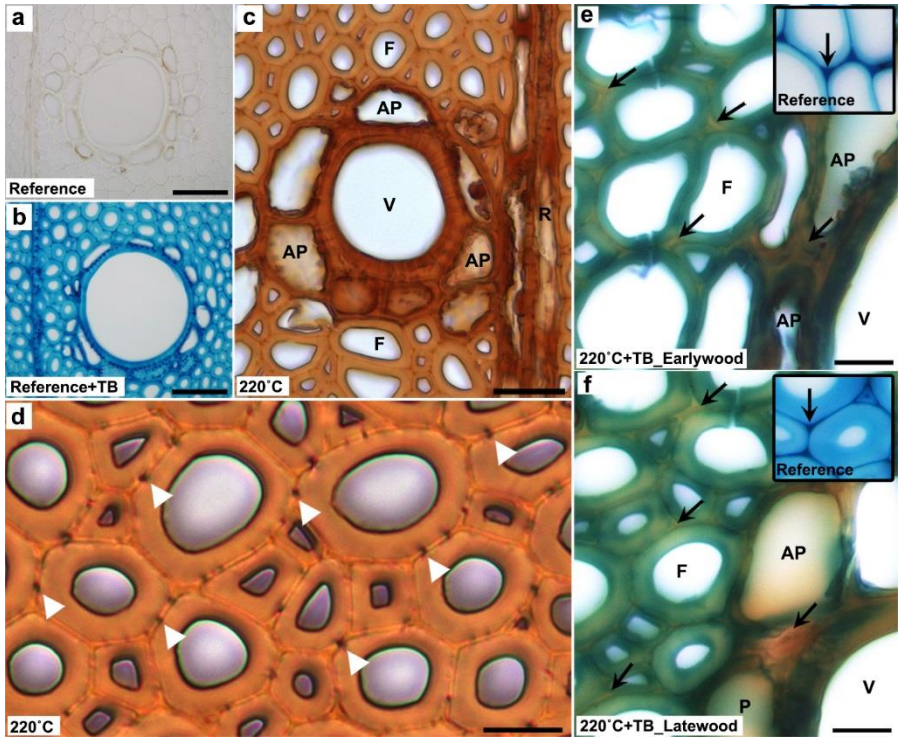

Figure 3. Changes in native cell color and structure in ash $TMW_{220°C}$. The color of thin sections shifted from white in reference (a) to orange/reddish in $TMW_{220°C}$ (c, d), with stronger coloration in vessel (V), axial (AP)/ray (R) parenchyma cells and CMLcc regions than fibres (F) (c). Note the formation of small cracks in latewood fibres of $TMW_{220°C}$ (arrowheads in d). After staining with toluidine blue (TB), the color in secondary cell walls and CMLcc regions (arrows in e, f) shifted from blue/dark blue in reference (b, insets in e, f) to green/orange-reddish in $TMW_{220°C}$ (e, f), respectively. Scale bars = 50 µm (a−c, e), 10 µm (d−f).

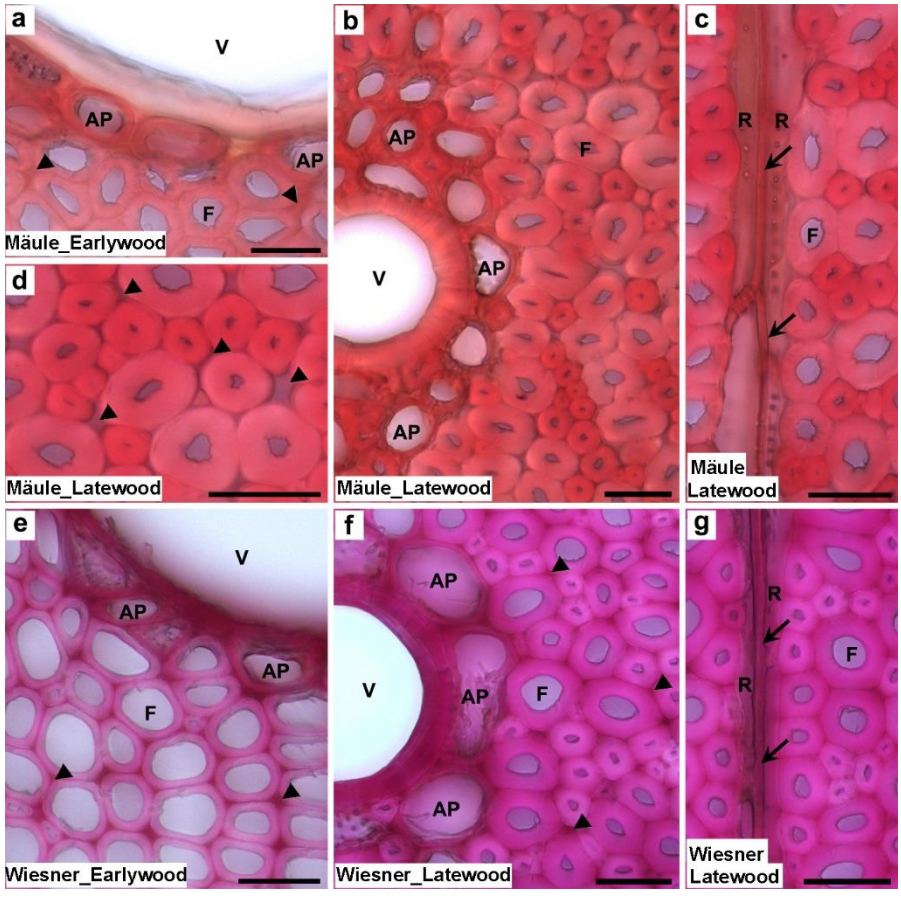

Figure 4. Lignin distribution in ash reference wood. Vessel (V) cell walls in earlywood (a) showed weaker Mäule reaction than in latewood (b). Axial/ray parenchyma cells (AP/R) revealed stronger Mäule reaction than fibres (a−c). Mäule reaction in large fibres (F) was weaker than that in small fibres (b−d). CMLcc regions showed stronger Mäule reaction in earlywood than latewood (arrowheads in a, d). Wiesner reaction was stronger in vessels, axial/ray (bi-/triseriate, arrows) parenchyma cells and CMLcc regions (arrowheads) than fibres (e−g). Scale bars = 25 µm.

As a result, the total lignin content, rather than the ratio of S/G lignin determined the color changes between cell types and between cell wall regions by thermal treatment. For example, no significant difference was found between early- and latewood vessels and between large and small fibre classes in TMWs (Figure 3c, d) even though these cells showed variations in the ratio of S/G lignin (Figure 4a−d). In contrast, the stronger Wiesner reaction (i.e. higher lignin content) observed in vessels and parenchyma cells (Figure 4e−g) was positively correlated with the stronger orange/reddish in these cells than fibres (Figure 3c).

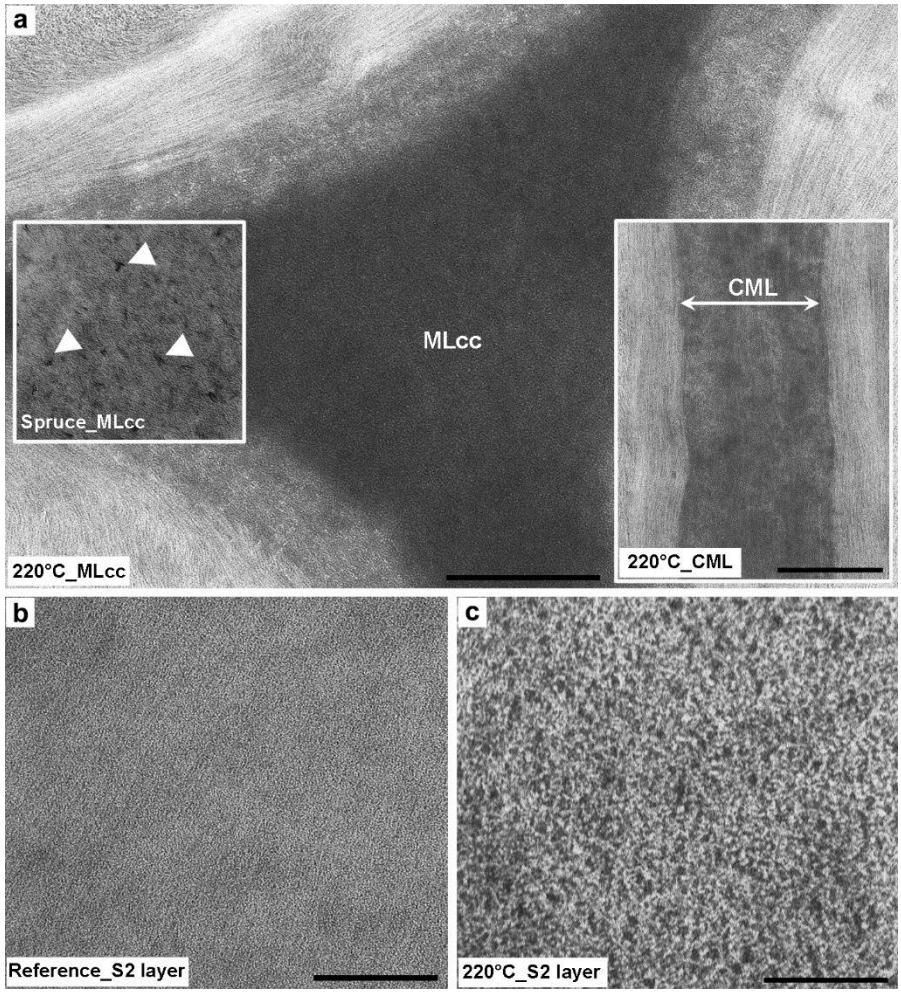

Figure 5. Changes in ultrastructure and lignin distribution in fibres of ash $TMW_{220°C}$. Stained with $KMnO_4$. Electron dense particles, observed in CMLcc regions of softwood $TMW_{220°C}$ (arrowheads in left inset), were not found in CMLcc regions of ash $TMW_{220°C}$ (a, right inset). Typical lamellar structure in the fibre S_2 layer of ash reference (b) was not detectable in $TMW_{220°C}$ (c). Note formation of large dark lignin aggregates in $TMW_{220°C}$ (c). Scale bars = 500 µm (a), 100 nm (b, c).

After staining with $KMnO_4$, ash $TMW_{220°C}$ displayed stronger staining intensity than the reference. As outlined for softwoods (spruce, fir), such a high staining intensity may be caused by the relative increase in lignin content through degradation of other polysaccharides and polycondensation reactions of lignin with other cell wall components in TMWs. Electron dense particles, observed in CMLcc regions of softwood $TMW_{220°C}$ (Figure 2g, left inset in Figure 5a), were not found in ash $TMW_{220°C}$ (Figure 5a and right inset). Interestingly, rather large dark staining lignin aggregates (with $KMnO_4$) were

apparent in the fibre S_2 layer of ash $TMW_{220°C}$ (Figure 5c), but were not detectable in ash reference (Figure 5b). These lignin aggregates in $TMW_{220°C}$ disrupted ordered lamellar structures of the fibre S_2 layer formed between lignin and cellulose microfibrils. The ultrastructural changes outlined above are consistent with published results on changes in lignin content, porosity, pore-size distribution, and reorganization of crystalline cellulose by thermal treatment (Pfriem *et al.*, 2010; Esteves & Pereira, 2009).

4 Decay resistance of Termovuoto treated softwoods and hardwoods

4.1 Decay resistance of soft- and hardwood TMWs to brown- and white-rot fungi

Mass loss (ML) data and durability classes (i.e. 1−5) of both reference and TMWs of two softwoods (spruce and fir treated at 160−220℃ for 4 h) and two hardwoods (ash and beech treated at 190−220℃ for 3 h) exposed to brown- and white-rot fungi are shown in Tables 1 and 2, respectively.

Table 1. Mass loss data (obtained from four replicates) of spruce and fir untreated reference and TMWs exposed to fungi indicated. Data in parenthesis indicates the durability classes of reference and TMWs after 18 weeks exposure.

Fungus	Untreated		160℃		180℃		200℃		220℃	
	Spruce	Fir	Spruce	Fir	Spruce	Fir	Spruce	Fir	Spruce	Fir
P. placenta	50.0	48.2	57.0	48.3	46.2	37.8	40.6	38.8	22.0	23.6
(BR)	(5)	(5)	(5)	(5)	(5)	(4)	(4)	(4)	(3)	(3)
P. sanguineus	55.6	59.1	64.6	61.2	-	-	44.8	49.0	23.6	29.2
(WR)	(5)	(5)	(5)	(5)			(4)	(4)	(3)	(3)

The SD of the mass loss data are less than ±5%

For spruce and fir TMWs, the mass loss decreased with increasing temperature over the range 180 to 220℃ (Table 1). Although small differences existed in mass loss at a certain treatment temperature, the two softwoods exhibited almost the same durability classes. In addition, there was no notable difference in durability class between the brown- (*P. placenta*) and white-rot (*P. sanguineus*) fungi used for either species of wood (Table 1). After 18 weeks of fungal exposure, the TMWs treated at and below 200℃ ($TMW_{160–200℃}$) showed durability class 4 (i.e. slightly durable) or 5 (i.e. non-

durable) in both spruce and fir, regardless of fungal species. When the treatment temperature reached 220℃, spruce and fir TMWs showed more than 50% reduction in mass loss compared to untreated reference wood, and the durability was classified as class 3 (i.e. moderately durable). Consequently, for the Termovuoto process, spruce and fir wood should be treated at temperatures higher than 200℃ to have a considerable improvement in decay resistance.

Table 2. Mass loss data (obtained from four replicates) of ash and beech untreated reference and TMWs exposed to the fungi indicated. Data in parenthesis indicates the durability classes of reference and TMWs after 18 weeks exposure.

Fungus	Untreated		190℃		200℃		210℃		220℃	
	Ash	Beech	Ash	Beech	Ash	Beech	Ash	Beech	Ash	Beech
P. placenta	29.6	54.0	25.5	57.7	14.4	53.1	3.1	30.7	0.6	11.7
(BR)	(5)	(5)	(4)	(5)	(3)	(5)	(1)	(3)	(1)	(2)
G. trabeum	55.5	62.7	38.9	58.5	32.4	57.2	10.3	25.1	6.9	13.7
(BR)	(5)	(5)	(4)	(5)	(3)	(5)	(2)	(3)	(1)	(2)
P. sanguineus	37.3	55.0	46.3	63.4	32.9	54.6	27.8	32.5	7.0	11.8
(WR)	(5)	(5)	(5)	(5)	(4)	(5)	(4)	(3)	(2)	(2)
P. radiata	29.0	28.3	36.3	34.9	25.9	30.8	14.3	21.1	6.6	12.7
(WR)	(5)	(5)	(5)	(5)	(4)	(5)	(3)	(4)	(2)	(3)

The SD of the mass loss data are less than ±5%

The mass loss of ash and beech TMWs exposed to brown rot fungi *P. placenta* and *G. trabeum* decreased when the treatment temperature reached 190℃ or above (Table 2). However, such a decreasing trend in mass loss was not observed when ash and beech TMWs were exposed to the white rot fungi *P. sanguineus* and *P. radiata* (Table 2). Instead, ash and beech $TMW_{190℃}$ showed a slight increase in mass loss compared to references (Table 2). After 18 weeks, the durability class of ash and beech $TMW_{220℃}$ ranged between 1 and 2 after exposure to brown rot fungi *P. placenta* and *G. trabeum* and ranged between 2 and 3 when subjected to white rot fungi *P. radiata* and *P. sanguineus* (Table 2), indicating higher decay resistance against brown- than white-rot fungi in hardwood TMWs at high treatment temperatures.

Overall, ash TMWs showed higher decay resistance than beech TMWs, no matter the temperature or which kind of fungal species was used (Table 2). For example, ash $TMW_{200℃}$ showed the same durability as beech $TMW_{210℃}$ (class 3) against two brown rot fungi. Windeisen and Wegener (2009) characterized the chemical composition of ash and beech $TMW_{200℃}$ and identified that there is only a difference in the amount of free phenolic groups between the two hardwood species. This suggests that the anatomical and structural differences

between ash and beech wood, especially the vessel arrangement may be closely related to the higher durability of ash TMWs than that of beech TMWs. Ash (*Fraxinus excelsior* L.) is a ring-porous hardwood with the largest vessels located in earlywood and small vessels distributed evenly in latewood. In contrast, beech (*Fagus sylvatica* L.) is a diffuse-porous hardwood which has relatively small diameter vessels distributed evenly across both early- and latewood. Presumably, a similar situation may exist for other thermal modification processes with the wood anatomy and ratio between cellular elements and thereby native chemistry playing an important role.

4.1.1 Comparison of decay resistance between soft- and hardwood TMWs

A clear difference in decay resistance between soft- and hardwood TMWs was observed (Tables 1, 2). Mass losses due to *P. placenta* and *P. sanguineus* attack were higher for softwoods (spruce, fir) than for ash (hardwood), with the difference even more evident at higher treatment temperatures. Beech $TMW_{200°C}$ gave higher mass loss than softwoods with exposure to the brown rot fungus *P. placenta* during the whole decay period, but had similar mass loss as softwoods when they were decayed by *P. sanguineus*. By raising the treatment temperature to 220℃, the decay resistance of beech $TMW_{220°C}$ was considerably improved with lower mass loss than softwoods in the presence of *P. placenta* or *P. sanguineus* (Figures 1, 2; Paper III). Ash wood seemed to be more resistant to decay after the Termovuoto process than the other wood species used (i.e. beech, spruce, fir). A considerable increase in durability occurred with ash wood treated at high temperatures (i.e. 210, 220℃) against the brown- and white-rot fungi tested. For example, ash $TMW_{210–220°C}$ did not show obvious decay (3.1 and 0.6% mass loss at $TMW_{210°C}$ and $TMW_{220°C}$, respectively) by *P. placenta* and was classified as class 1 (i.e. very durable).

According to previous studies, decay resistance of TMW is strongly correlated to the weight loss caused by the actual thermal treatment process (Chaouch *et al.*, 2010; Šušteršic *et al.*, 2010). Ferrari et al. (2012, 2013) compared the weight loss of hard- and softwoods caused by Termovuoto technology under different temperatures. Ash and beech wood showed an overall higher weight loss than softwoods. When the temperature was higher than 180℃, the weight loss of ash were much greater than softwoods. By increasing the temperature from 200 to 220℃, hardwoods (ash, beech) experienced an exponential increase in weight loss (twice as high at 220℃ than 200℃). This is consistent with our findings that the mass loss of beech TMWs, compared to softwoods, was higher at 200℃ and lower at 220℃. Present results also agree with those of Hakkou *et al*. (2006) who suggested that the

durability of heat treated beech wood starts to increase at 200℃ when weight loss due to thermal degradation occurs.

4.1.2 Changes in decay resistance of TMWs depending on temperature

Lower treatment temperatures apparently accelerated fungal decay. Spruce and fir $TMW_{160℃}$ were quite sensitive to attack by both brown- (*P. placenta*) and white-rot (*P. sanguineus*) fungi as suggested by slightly higher mass losses compared to reference especially for *P. sanguineus* (Table 1). Similar results have been also reported by Sivonen *et al.* (2003) who found that pine TMWs treated at 120 and 140℃ showed higher mass loss against *P. placenta* than TMWs treated at 100℃. Earlier results showed that depolymerized hemicelluloses without severe denaturalization and degradation are more readily consumed by fungi (Mazela *et al.*, 2004). In addition, sugar anhydrides, mono- and oligo- as well as polysaccharides may polymerize at lower temperatures (i.e. below 200℃) to form dextrin and other branched carbohydrates. Such carbohydrates may serve as substrates and become more accessible to fungal attack (Mazela *et al.*, 2004; Sivonen *et al.*, 2003).

Similar results were also shown by exposing ash and beech $TMW_{190℃}$ to the white rot fungi *P. sanguineus* and *P. radiata* (Table 2). However, this conclusion does not necessarily follow when brown rot fungi are used. Considering that white rot fungi are capable of degrading both polysaccharides and lignin and that brown rot fungi rapidly degrade carbohydrates and primarily only modify lignin (i.e. demethylation) and do not metabolize lignin to a great extent, we conclude that the elevated mass loss in the presence of white rot fungi presumably results from the thermo-vacuum treatment induced modification of the cell wall and lignin, which at low temperature treatments may allow better accessibility for lignolytic enzymes and non-enzymatic catalysts.

4.1.3 Comparison between soil block (AWPA E10-08) and agar block (EN 113) testing of hardwoods

Our findings from AWPA E10-08 soil block test were in agreement with data from the EN 113 agar block test (Table 3), with no notable difference in durability class of TMWs between the two tests. The mass loss and durability class achieved by thermal modification depended on the wood species, treatment temperature and test fungus. AWPA E10-08 and EN 113 also confirmed improved decay resistance of ash wood from Termovuoto treatment. Compared with spruce, fir and beech wood, ash is the best candidate for treatment by the Termovuoto process, which can attain the same durability level at lower treatment temperatures by using a shorter time.

Table 3. Comparison of durability classes between soil-block (AWPA E10-08) and agar-block test (EN 113) for hardwood TMWs.

Wood		Fungus	Decay test	Treatment temperature			
				190℃	200℃	210℃	220℃
Ash	Brown rot	*P. placenta*	EN 113	5	3	2	1
			AWPA E10-08	4	3	1	1
		G. trabeum	EN 113	3	2	1	1
			AWPA E10-08	4	3	2	1
	White rot	*T. versicolor*	EN 113	4	4	3	3
		P. sanguineus	AWPA E10-08	5	4	4	2
		P. radiata	AWPA E10-08	5	4	3	2
Beech	Brown rot	*P. placenta*	EN 113	5	5	2	1
			AWPA E10-08	5	5	3	2
		G. trabeum	EN 113	4	4	1	1
			AWPA E10-08	5	5	3	2
	White rot	*T. versicolor*	EN 113	5	5	4	3
		P. sanguineus	AWPA E10-08	5	5	3	2
		P. radiata	AWPA E10-08	5	5	4	3

Durability classes in EN 113 test are cited from Terziev (2014).

In summary, a wide spectrum of factors, including fungal species, treatment temperatures and wood species affect the decay resistance of Termovuoto treated TMWs. Mass losses indicate that there is a considerable improvement in decay resistance of TMWs with increasing temperature. After 18 weeks exposure to brown- and white rot fungi, wood samples treated at 200℃ or above showed improved decay resistance, as demonstrated by low mass loss. Wood samples treated at 220℃ show lowest mean values of mass loss and durability class (i.e. 1−3). The experiments based on Termovuoto treated TMWs against basidiomycetes (i.e. brown- and white-rot fungi) indicate that the results from the soil block test (AWPA E10-08) agree very well with those from the agar block (EN 113) approach. These results help us understand and evaluate the impact of thermal modification on the durability of wood samples by different approaches and contribute further for optimizing the Termovuoto process.

4.2 Decay resistance of soft- and hardwood TMWs to soft rot fungi

4.2.1 Comparison of decay resistance between soft- and hardwood TMWs

Figures 6 and 7 show the mass loss of Termovuoto treated hard- and softwood TMWs and corresponding reference woods exposed to *P. malorum*, *P. mutabilis* and *C. globosum* for different exposure periods.

Mass loss of hardwood TMWs was always less than that of the corresponding reference woods. Greatest decay resistance was observed with treatment at 220℃ for both ash and beech wood (Figure 6). Differences in the mass loss between fungal species tested were negligible with $TMW_{220℃}$, whereas values from reference and $TMW_{190–210℃}$ showed the order *P. mutabilis* > *C. globosum* >> *P. malorum*, emphasizing the variation in decay ability between fungal species.

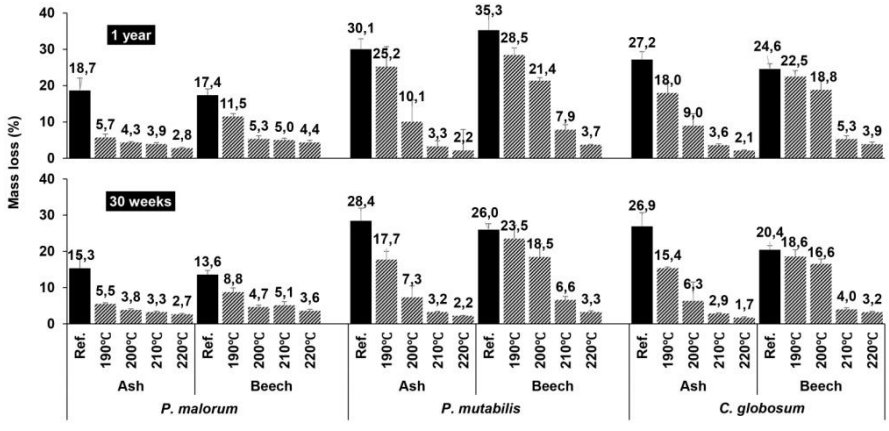

Figure 6. Mass loss (obtained from four replicates) of ash and beech (hardwoods) reference (Ref.) and $TMW_{190–220℃}$ exposed to soft rot fungi *P. malorum*, *P. mutabilis* and *C. globosum* over 1 year.

Interestingly, the patterns of mass loss between the two hardwoods were different. Ash wood showed similar or higher mass losses than beech in reference groups, but lower mass loss than beech in TMWs groups over the entire exposure period. The major reduction in mass loss (i.e. more than 50% reduction compared to reference) in relation to treatment temperature also differed between ash and beech TMWs. For example, after 30 weeks exposure to *P. mutabilis*, the major reduction in mass loss was detected at 200℃ for ash (ca. 66%) but was at 210℃ for beech (ca. 75%). The reduction in mass loss was only ca. 29% for beech $TMW_{200℃}$. These results indicate that thermal treatment causes different impacts on ash and beech wood. Similar differences were also detected in mass loss patterns against brown- and white-rot fungi

between ash and beech TMWs (See chapter 4.1; Gao *et al*., 2016). Thus, it can be assumed that natural variations in the anatomy (i.e. ash is a ring porous hardwood and beech is diffuse porous hardwood) and chemistry between ash and beech wood likely influence the difference in thermal behavior of wood, and therefore affect resistance of TMWs against soft rot fungi.

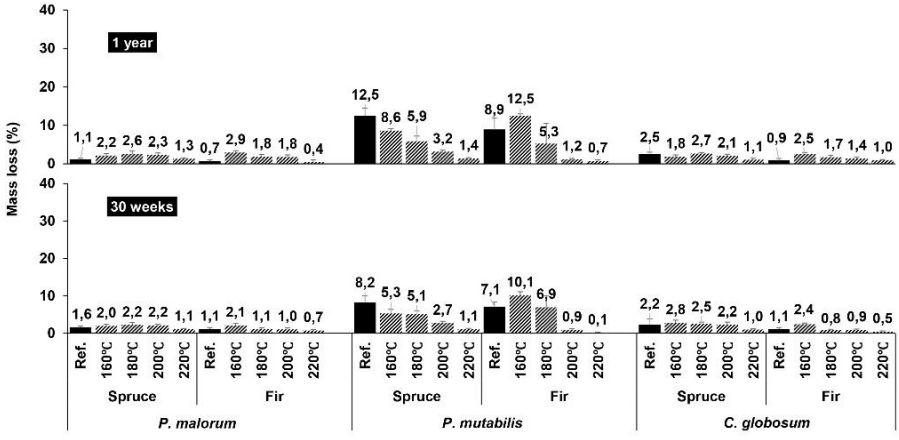

Figure 7. Mass loss (obtained from four replicates) of spruce and fir (softwoods) reference (Ref.) and $TMW_{160–220°C}$ exposed to soft rot fungi *P. malorum*, *P. mutabilis* and *C. globosum* over 1 year.

In contrast to hardwoods that were decayed quite rapidly by the soft rot fungi, softwoods showed much less mass loss during the same exposure period (Figure 7). Previous studies have shown that the slower rate of fungal degradation observed in softwoods is influenced strongly by the nature and composition of lignin that differs considerably from hardwoods (Singh *et al.*, 2006; Daniel & Nilsson, 1998). Ash and beech (hardwoods) have a lignin content of around 18−22%, while softwoods have a higher lignin content of 25−28%, implying a greater resistance of softwoods against soft rot fungi. In addition, softwood lignin is composed almost exclusively of G-lignin, which is assumed to have moderate resistance to soft rot fungi, whereas hardwood lignin is characterized by both G- and S-units that have variable distribution according to cell type and cell wall layer. For example, the fibre secondary cell walls in hardwoods (i.e. usually highly degraded by soft rot fungi) are primarily S-lignified. This suggests that natural varieties in lignin chemistry between soft- and hardwoods significantly affect the difference in decay resistance of TMWs. With increase in treatment temperature, the mass loss difference between hard- and softwoods is decreases, indicating that the impact

of initial differences in lignin chemistry between hard- and softwoods on the durability of soft rot decay also decreases.

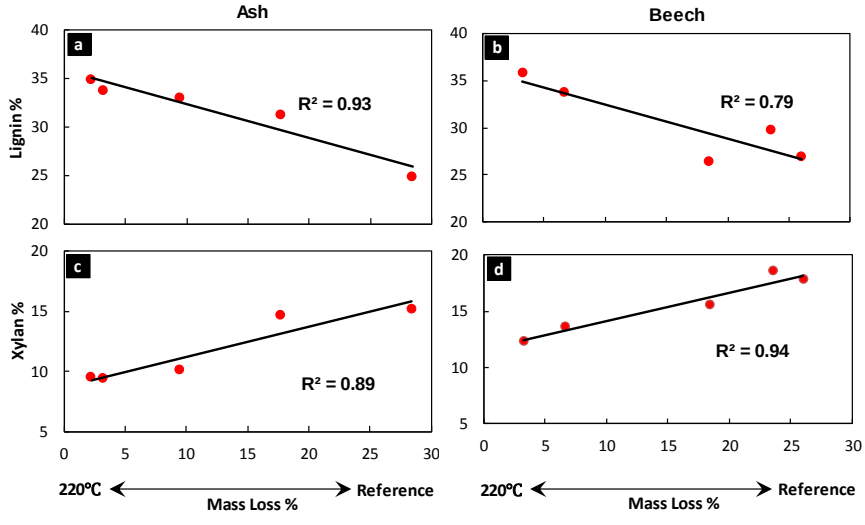

Figure 8. Correlation between changes in lignin/xylan content of TMWs and mass loss after 30 weeks decay by *P. mutabilis* (left to right 220, 210, 200, 190℃, reference). Mass loss showed positive and negative correlation with lignin (a, b) and xylan (c, d) content, respectively, in both ash (a, c) and beech (b, d) TMWs. Chemical data of lignin and xylan content are cited from Jebrane *et al.* (2016).

The reasons for mass loss decrease in TMWs treated at higher temperatures are still poorly understood. However, it is generally accepted that improved decay resistance of TMWs is related to changes in chemical components (Esteves & Pereira, 2009), such as the apparent increase in lignin content caused by a decrease of accessible polysaccharides (mainly hemicelluloses), which is apparently an important factor for soft rot decay. A recent study (Jebrane *et al.*, 2016) showed the Termovuoto process caused a relative increase in percentage of total lignin content for both ash and beech wood via a decrease in total xylan content (Figure 8). For example, when the treatment temperature for beech reached 210℃, a substantial increase in total lignin content (ca. 25%) was shown, while the xylan content decreased dramatically (ca. 23%) relative to the reference (Figure 8b, d). The major change in lignin and xylan content occurred at temperatures between 190 and 200℃ in the two hardwoods (Figure 8). Mass loss from the soft rot decay test showed a negative linear correlation with the lignin content (Figure 8a, b) and a positive relation with the xylan content (Figure 8c, d) in the two hardwoods. The percentage of glucan (i.e. cellulose) fluctuated between 43−50% for ash and 38−45% for

beech, and no significant correlation with mass loss was found (Jebrane *et al.*, 2016). These results suggest that even if the cellulose content does not change remarkably, wood blocks with lower hemicelluloses and higher lignin content are more durable to soft rot decay (i.e. by *P. mutabilis*). It should be noted that the results of mass loss and chemical changes discussed are from gross analyses of wood blocks. However, the patterns of polymer distributions at tissue and individual cell levels (e.g. fibre, parenchyma cell, vessel) vary considerably in hardwoods as outlined earlier in this thesis and in Paper IV.

4.2.2 Correlation between changes in moisture content of TMWs and mass loss

Since TM of wood commonly decreases the equilibrium moisture content of wood, and that soft rot fungi typically prefer to attack high moisture containing wood (Esteves & Pereira, 2009; Daniel, 2003), the moisture content (MC) of the soil (i.e. used in the test flasks) was adjusted monthly to around 70−75%, and the MC of wood blocks was monitored during the entire decay period. When the treatment temperature increased from 190 to 220℃, the MC of hardwood TMWs (ash, beech) decreased and was positively correlated with mass loss for all three soft rot fungi tested (Figure 9a−c). By contrast, the MC of softwood TMWs (spruce, fir) were similar to the reference at all temperatures (160−220℃), and were higher than those of hardwood reference and TMWs (Figure 9d−f). These results suggest that the ability for water absorption in TMWs may differ significantly between hard- and softwood TMWs. However, we are not sure whether the decreased MC in hardwood TMWs actually has an effect on the inhibition or delay of soft rot decay in TMWs, since there is no documented information about the optimal levels of MC for soft rot growth in TMWs. Laboratory decay tests generally allow a final MC of decayed wood blocks in the range 25−80% (EN 113, 2004). In our work, although the MC of decayed samples is lower than that of the reference, we consider the value ranges 50−60% in $TMW_{190–220℃}$ are sufficient for the growth of the soft rot fungi species used. Another important point to stress is that the decrease in mass loss of hardwood TMWs is likely induced by changes in other factors along with change of MC, such as the apparent increase in lignin content as outlined above. Weak positive correlation between changes in mass loss and MC in softwood TMWs decayed by soft rot fungi may also reflect this aspect (Figure 9d−f). For example, lower mass loss in TMWs than reference may simply reflect the apparent increase in relative lignin content after thermal treatment, since TMWs and reference showed similar levels of high MC in softwoods.

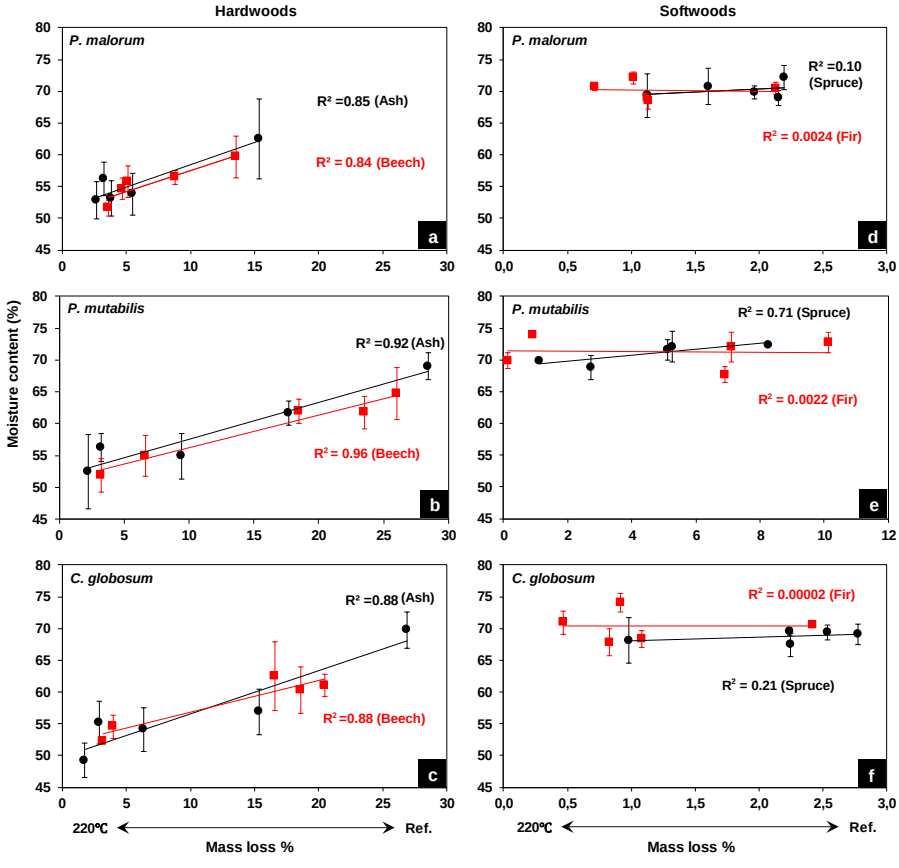

Figure 9. Correlation between changes in moisture content (MC, obtained from four replicates) of TMWs and mass loss after 30 weeks decay (left to right 220, 210, 200, 190°C, reference). Hardwoods (ash, beech) showed decreased MC in TMWs compared to reference (Ref.), with greater decrease at higher temperature (a−c), while softwoods (spruce, fir) revealed similar MC between TMWs and reference. Note strong and weak positive correlation between changes in MC and mass loss in hardwoods and softwoods, respectively.

5 White rot decay patterns of Termovuoto treated softwoods and hardwoods

From decay resistance and durability studies (Section 4), it was apparent that white rot fungi caused significant mass loss of TMWs and even attacked wood treated at the highest temperature used (i.e. 220°C; see Tables 1, 2). From the known changes induced in wood during thermal treatment, such as loss and redistribution of hemicelluloses, relative increase in lignin content and condensation and modification of cell wall ultrastructure (Sections 1−3), it can be hypothesized that TMWs may show differences in morphological features of the decay process compared with reference against white rot fungi. To elucidate these aspects, micro- and ultrastructural analyses were carried out on untreated (reference) and TMWs exposed to the white rot fungus *Pycnoporus sanguineus* for 10 weeks using microscopy techniques. In particular, the decay process in $TMW_{220°C}$ was studied in comparison with reference. For more specific details see Paper V.

5.1 Observations of *P. sanguineus* degraded softwood TMWs

Overall, reference and TMWs in the two softwoods (spruce and fir) were colonized similarly with hyphal advancement through the open ends of tracheids and along the rays with growth and development in the cell lumina. Figure 10 shows examples of the decay of tracheids from reference and $TMW_{220°C}$. For both reference and $TMW_{220°C}$, earlywood (EW) tracheids showed greater resistance of tangential (Ta)- than radial (Ra) cell walls, while latewood (LW) tracheids revealed opposite pattern (Figure 10a, b). Tangential longitudinal sections of reference and $TMW_{220°C}$ showed colonization of the cell lumina of tracheids and rays (i.e. ray parenchyma, ray tracheids) with decay initiated from bordered pits by enlargement of the pit borders to form large bore holes (Figure 10c, d).

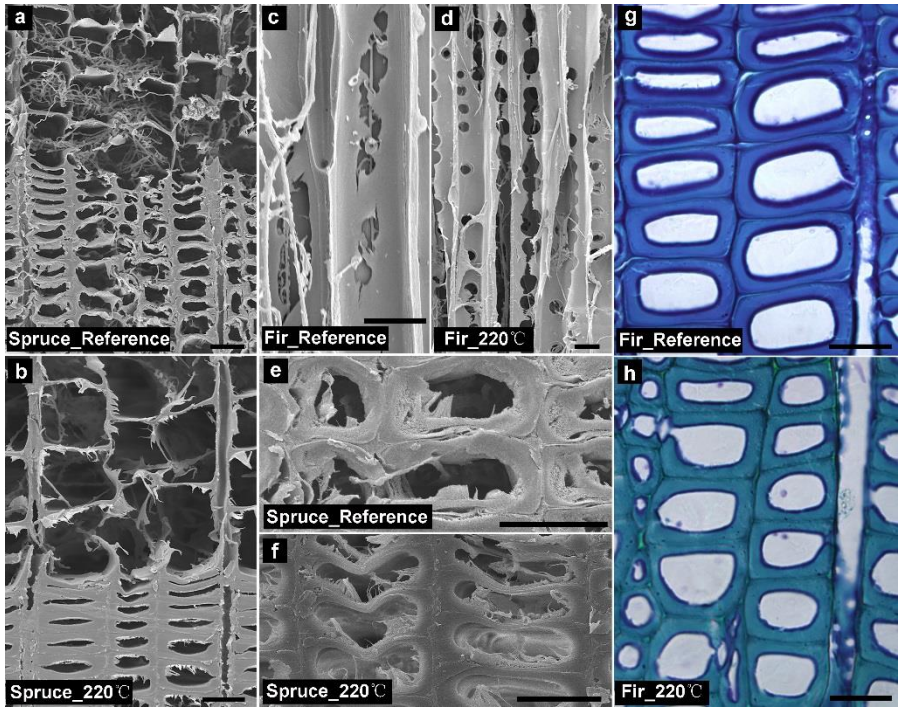

Figure 10. Softwood reference and $TMW_{220°C}$ degraded by *P. sanguineus*. Both reference and $TMW_{220°C}$ showed similar decay patterns; more severe degradation in early- than latewood (a, b), decay from the pits by enlarging the size of the border to form bore holes (c, d), delignification and decay of tracheid secondary cell walls as evidenced by a bright concentric zone with a 'spongy-like' cell wall structure from SEM observations (e, f) and a dark purple colored decayed cell walls with toluidine blue staining (g, h). Scale bars = 25 μm (a−f), 100 μm (g, h).

Principle decay of tracheid cell walls by *P. sanguineus* in both reference and $TMW_{220°C}$ was by slow erosion of the surrounding lumen cell wall producing concentric delignification zones that progressed outwards across cells, reflecting attack in "time and space". The concentric zones were readily visible using light microscopy (LM) and particularly distinct in latewood tracheids after staining with toluidine blue (Figure 10g, h; arrows; Paper V). Using SEM, the zones were visible as a less dense and more open layer (i.e. presumably enhanced during sectioning) surrounding the cell lumina (Figure 10e, f; arrows). In more advanced phases of hyphal attack, delignification in the concentric zones passed across middle lamella regions into adjacent tracheids in both reference and $TMW_{220°C}$. The development of concentric zones in wood cells is a characteristic feature of selective (i.e. preferential) white rot decay of wood cells including *P. sanguineus* (Kim *et al.*, 2015a; Daniel, 1994; Daniel, 2014).

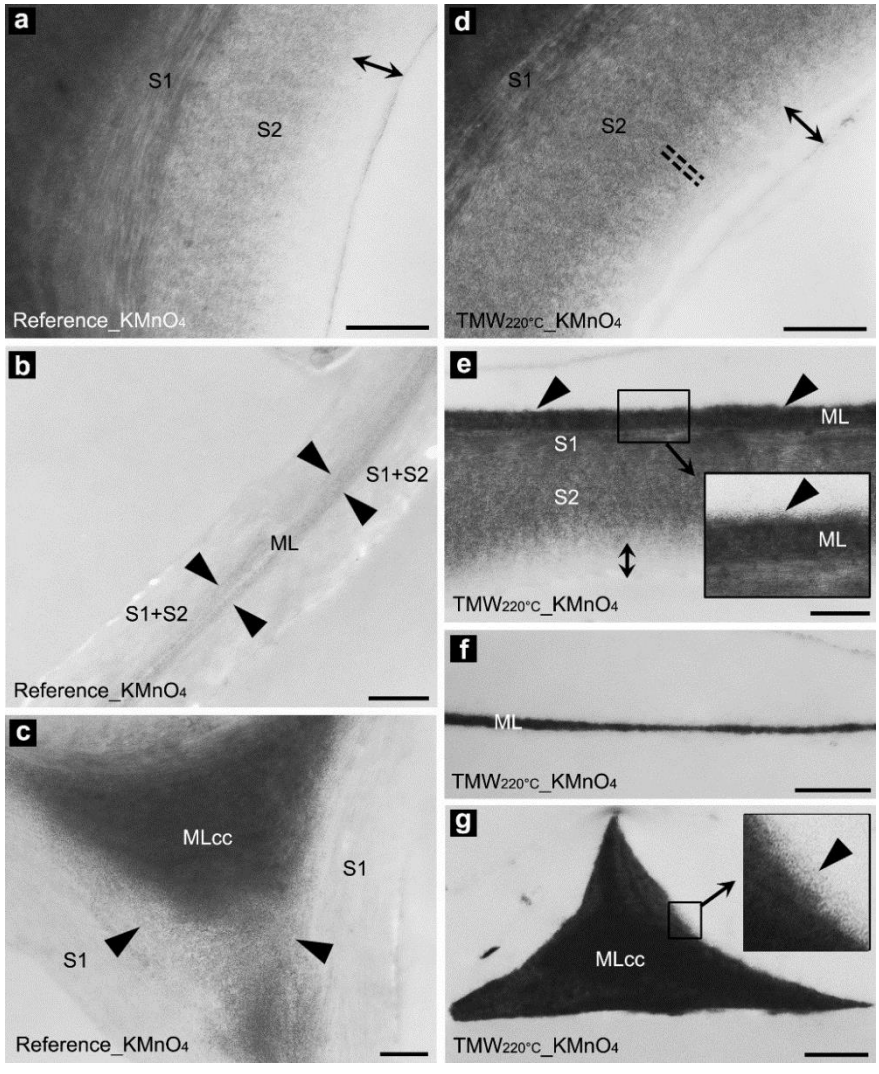

Figure 11. Decay of spruce tracheids by *P. sanguineus*. Stained with $KMnO_4$. Reference (a−c) and $TMW_{220°C}$ (d−g) showing progressive delignification from the lumen outwards and formation of prominent delignified zones (double headed arrows) in secondary cell walls (a, d). Note formation of the transition zone from delignified- to lignified cell wall regions in $TMW_{220°C}$ (dashed lines in d) and the relatively wider area of delignification (arrowheads) in CMLcc regions of reference (b, c) than those of $TMW_{220°C}$ (e, g). A residual ML region after complete degradation of tracheid secondary cell wall (f) was also detected only in $TMW_{220°C}$. Scale bars = 500 nm.

TEM studies combined with $KMnO_4$ staining for lignin on spruce reference and $TMW_{220°C}$ showed further details on the formation of concentric delignification zones in tracheid cell walls (Figure 11). As observed in LM and

SEM, both reference and $TMW_{220°C}$ showed progressive delignification in tracheid cell walls from the lumen surface outwards, followed by formation of prominent delignified zones (double headed arrows in Figure 11a, d). However, delignification in $TMW_{220°C}$ contrasted with reference in that the transition zone from delignified- to lignified cell walls (dashed lines in Figure 11d) in which preferential lignin degradation occurred in tracheid cell walls was much thinner and more distinct. A similar difference was also detected in the compound middle lamella (CML/ML) including middle lamella cell corner (MLcc) (CMLcc) regions. $TMW_{220°C}$ (arrowheads in Figure 11e, g) showed much narrower delignified zones in these regions compared to reference (arrowheads in Figure 11b, c). These results suggest that delignification of tracheids in $TMW_{220°C}$ occurs in much narrower area than that in reference. The ML remaining after complete degradation of tracheid secondary cell walls in $TMW_{220°C}$ supports this idea (Figure 11f).

Consequently, results demonstrate that characteristic features of decay in softwoods by *P. sanguineus* including colonization of fungal hyphae, general variations in decay between early- and latewoods and preferential delignification are not changed by thermal modification. However, results suggest that delignification in tracheids is slower in $TMW_{220°C}$ than reference, thereby leading to delay of tracheid degradation in $TMW_{220°C}$.

TEM immunogold labeling combined with $KMnO_4$ staining showed that the process of hemicellulose removal by *P. sanguineus* is similar between reference and $TMW_{220°C}$ (Figure 12a, b; Paper V). Both samples showed presence of abundant heteroxylan and heteromannan epitopes in the electron-lucent decay zones (i.e. where lignin had been degraded) (double headed arrows in Figure 12a, b). These results indicate that lignin is preferentially degraded prior to complete degradation of hemicelluloses by *P. sanguineus* during progressive decay in both reference and $TMW_{220°C}$. At the same time, results indicate that thermal modification does not affect significantly the temporal degradation patterns of lignin and hemicelluloses in spruce tracheids.

Unlike reference (Figure 12c), $TMW_{220°C}$ sections stained with uranyl acetate frequently showed greater electron density layers in decayed tracheid cell walls (arrowheads in Figure 12d, e), probably indicating the transition zone from delignified- to lignified cell walls as evidence by $KMnO_4$ staining (Figure 11d). $TMW_{220°C}$ also showed similar positive uranyl acetate staining across tracheid cell walls including decayed cell walls (double headed arrows in Figure 12d, e), while reference revealed positive and almost negative staining in the un-decayed- and decayed cell walls, respectively (Figure 12c). Based on the principle that an aqueous solution of uranyl acetate does not stain crystalline cellulose but stains amorphous phase cellulose (Heyn, 1966), the

degree of crystallinity of cellulose remaining after delignification by *P. sanguineus* may be lower in $TMW_{220°C}$ than reference, thereby increasing the intensity of staining in delignified cell walls. However, the result should be further investigated since the change in cellulose crystallinity by thermal modification is a controversial issue and results vary depending on treatment temperature (reviewed by Esteves & Pereira, 2009). Formation of higher electron dense layers in $TMW_{220°C}$ also suggests that substances that readily react with uranyl acetate may be accumulated in transition zones, most likely protein-based such as ligninolytic enzymes. For example, several previous studies have reported the concentration of lignin/Mn peroxidases and laccase at the interface between degraded (i.e. delignified) and non-degraded cell walls (Daniel *et al.*, 2004; Daniel, 1994; Daniel *et al.*, 1991; Daniel *et al.*, 1989; Srebotnik *et al.*, 1988).

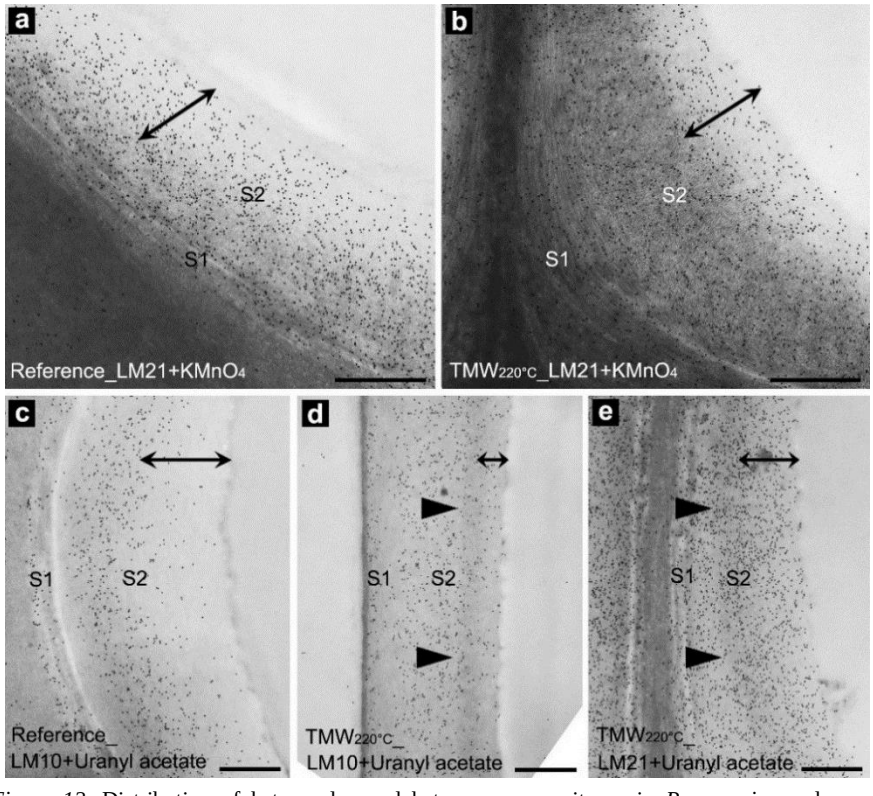

Figure 12. Distribution of heteroxylan and heteromannan epitopes in *P. sanguineus* decayed spruce tracheid cell walls. Reference (a) and $TMW_{220°C}$ (b) stained with $KMnO_4$ showing abundant LM21 heteromannan epitopes in delignified cell wall layers (double headed arrows). Reference (c) and $TMW_{220°C}$ (d, e) stained with uranyl acetate showing almost negative and positive staining in the delignified cell walls (double headed arrows), respectively. Note localization of LM10 heteroxylan (c, d) and LM21 heteromannan (e) epitopes in delignified cell

walls and formation of more electron dense layers (arrowheads in d, e) in $TMW_{220°C}$. Scale bars = 500 nm.

5.2 Observations of *P. sanguineus* degraded hardwood TMWs

Fungal colonization of hardwood (ash, beech) reference and TMWs was in principle similar with hyphal growth developing within the wood structure from the vessels and rays as shown in examples from reference and $TMW_{220°C}$ (Figure 13). Initial decay and colonization in both reference and TMWs was characterized by prominent development of bore holes between fibre-fibre/vessel-vessel/fibre-ray parenchyma cells and scarce development between ray parenchyma cells (Figure 13c, d). Hyphal colonization in the cell lumina of fibres from where attack of the secondary cell walls was initiated and earlier decay of early- than latewood fibres were also detected in both reference and TMWs (Figure 13a, b). Vessels and ray/axial parenchyma cells showed greater and lower decay resistance than fibres in ash (Figure 13a, b) and beech (Figure 13e, f) respectively in both reference and TMWs.

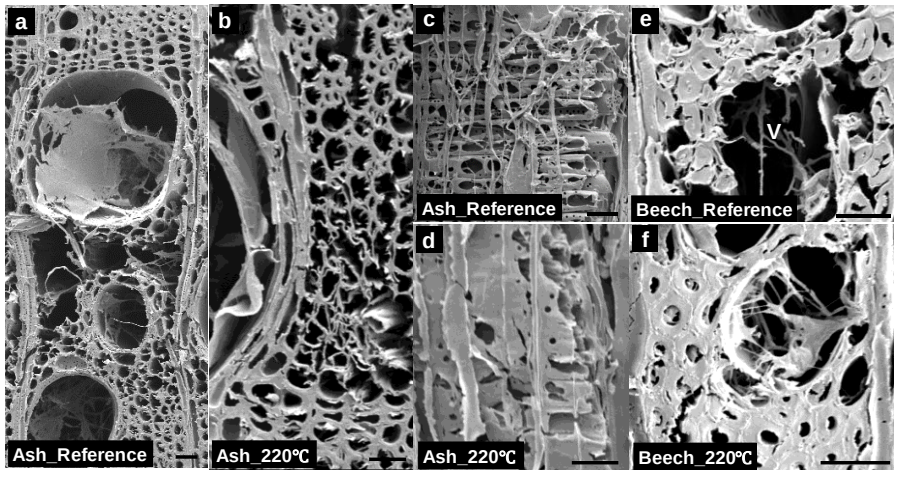

Figure 13. P. sanguineus degraded ash (a−d) and beech (e, f) reference and $TMW_{220°C}$. More severe degradation in early- than latewood (a, b) and formation of bore holes was apparent (c, d). Note the lower and greater decay in vessels than fibres in ash (a, b) and beech (e, f) respectively in both reference and $TMW_{220°C}$. Scale bars =25 µm.

Like softwood tracheids, preferential delignification was apparent, followed by cell wall erosion of fibres in both reference and TMWs. Delignification was also typically initiated from hyphae in the fibre cell lumina and developed outwards into the secondary cell walls frequently passing through ML into adjacent fibres (Figure 14a). Interestingly, fibres unlike tracheids showed a notable difference in staining patterns of decayed cell walls depending on

treatment temperature (Figure 14b–g). A distinct concentric line between lignified- and delignified cell walls was frequently detected in $TMW_{200–220°C}$ and particularly $TMW_{220°C}$ (Figure 14d–g). This line presumably reflects the narrow transition zones observed in $TMW_{220°C}$ with TEM (see below). The intensity of safranin staining in delignified cell wall regions was stronger than lignified cell walls in reference (Figure 14b) but changed gradually as treatment temperature increased (Figure 14c–e) and finally showed lower intensity in $TMW_{220°C}$ (Figure 14f, g). This result may indicate that the cell wall structure in the delignified zone becomes more compact as the treatment temperature increased and thus the staining solution cannot easily penetrate into this zone (i.e. difficulty of stain adsorption).

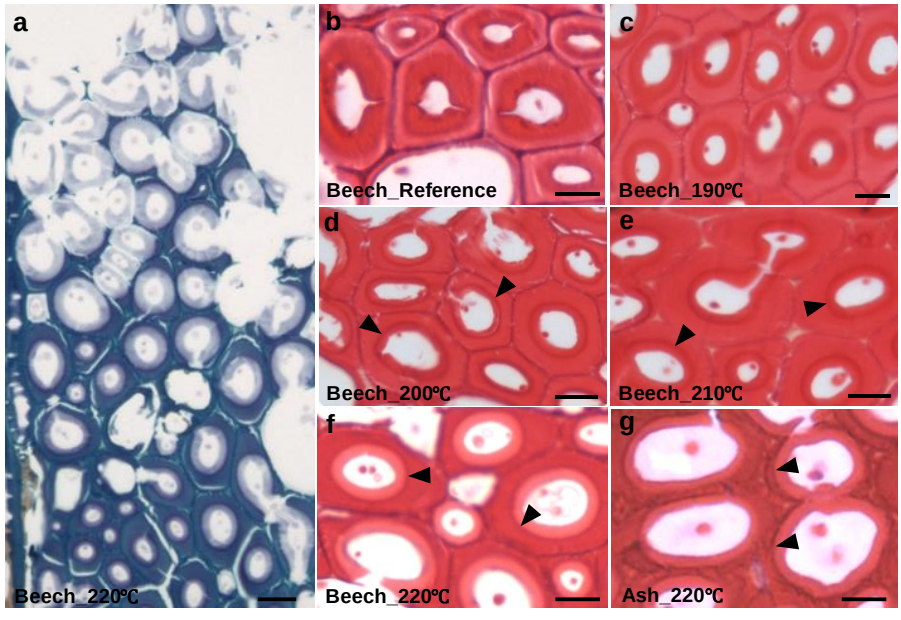

Figure 14. P. sanguineus degraded fibres of beech (a–f) and ash (g) $TMW_{220°C}$. Stained with toluidine blue (a) and safranin (b–g). Preferential delignification of fibres, followed by cell wall erosion (a). Note changes in intensity of safranin staining in delignified fibre cell walls and formation of concentric lines (arrowheads) with increase in temperatures (d–g). Scale bars =25 µm.

Characteristic features of delignification in secondary cell walls of ash fibres were similar to those in spruce tracheids, regardless of thermal modification. Both reference (Figure 15a) and $TMW_{220°C}$ (Figure 15d) showed progressive delignification in fibre cell walls from the lumen surface outwards, followed by formation of prominent delignified zones (double headed arrows in Figure 15a, d). The only difference was the width of transition zones that were overall narrower in ash fibres (Figure 15d) than spruce tracheids (Figure

11d). Features of delignification in CMLcc regions of ash fibres were also similar to those of spruce tracheids. Delignification in CMLcc regions of fibres progressed gradually from the outermost layer inwards in $TMW_{220°C}$ (arrowheads in Figure 15e, f), followed by formation of very narrow transition zones, but occurred in a relatively wider area in reference (arrowheads in Figure 15b, c).

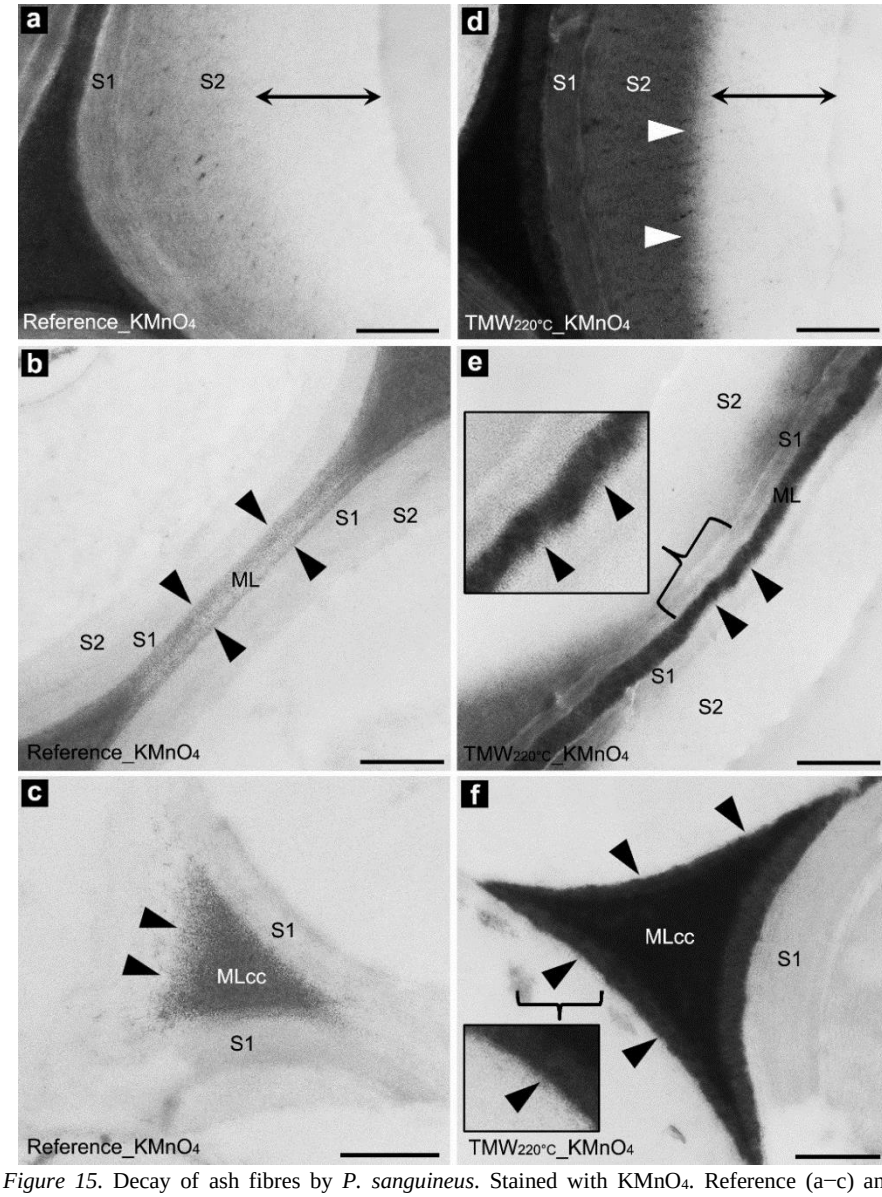

Figure 15. Decay of ash fibres by *P. sanguineus*. Stained with $KMnO_4$. Reference (a−c) and $TMW_{220°C}$ (d−f) showing progressive delignification from the lumen outwards and formation of

prominent delignified zones (double headed arrows) in secondary cell walls (a, d). Note formation of the narrow transition zone from delignified- to lignified cell walls in $TMW_{220°C}$ (arrowheads in d) and the relatively wider area of delignification (arrowheads) in CMLcc regions of reference (b, c) than those of $TMW_{220°C}$ (e, f). Scale bars = 500 nm.

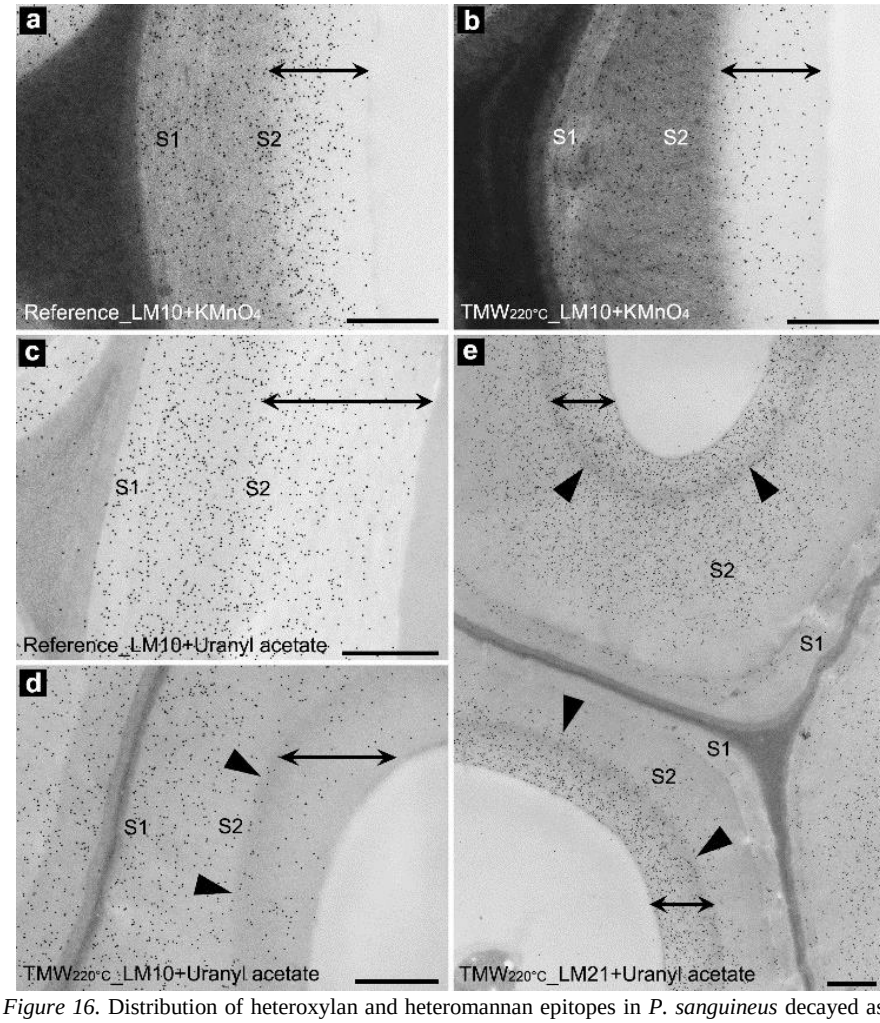

Figure 16. Distribution of heteroxylan and heteromannan epitopes in *P. sanguineus* decayed ash fibre cell walls. Reference (a) and $TMW_{220°C}$ (b) stained with $KMnO_4$ showing abundant LM10 heteroxylan epitopes in delignified cell wall layers (double headed arrows). Reference (c) and $TMW_{220°C}$ (d, e) stained with uranyl acetate showing almost negative and positive staining in delignified cell walls (double headed arrows), respectively. Note localization of LM10 heteroxylan (c, d) and LM21 heteromannan (e) epitopes in delignified cell walls and formation of more intense electron dense layers (arrowheads in d, e) in $TMW_{220°C}$. Scale bars = 500 nm.

Results of TEM immunogold labeling of degraded ash fibres were also similar to those in spruce tracheids. Abundant heteroxylan and heteromannan epitopes were detected in delignified fibre cell wall regions (as evidenced by the lack of $KMnO_4$ staining) of reference and $TMW_{220°C}$ (Figure 16a, b). As outlined for spruce tracheids, this result indicates that the fundamental decay process of ash fibres by *P. sanguineus* in relation to preferential delignification prior to degradation of hemicelluloses is not affected by thermal modification. Development of more intense electron dense layers (arrowheads in Figure 16d, e) and positive uranyl acetate staining in decayed fibre cell walls in $TMW_{220°C}$ (double headed arrows in Figure 16d, e) that were not detected in reference (Figure 16c) also suggest modification in the degree of cellulose crystallinity in delignified fibre cell walls and accumulation of protein-based substances in transition zones following thermal modification.

5.3 Section conclusions

Results demonstrate that thermo-vacuum modification does not affect characteristic features of preferential delignification by *P. sanguineus* in spruce tracheids (softwood) and ash fibres (hardwood), regardless of treatment temperatures. However, results suggest that the delignification process in tracheids and fibres by *P. sanguineus* may be delayed in TMWs, particularly at high treatment temperature. Formation of narrower transition zones in $TMW_{220°C}$ than reference likely reflects the difference in delignification process following thermal modification. At present, there are two plausible explanations in relation to delay of delignification in TMWs by *P. sanguineus*. The first is delay of delignification by an apparent increase of lignin content following degradation of polysaccharides (mostly hemicelluloses) and modification of the lignin structure, particularly increase of condensation in $TMW_{220°C}$. This expectation is based on the assumption that *P. sanguineus* produces the same qualitative and quantitative ligninolytic enzymes and free radicals in reference and $TMW_{220°C}$. Since TMWs do not contain fungicides in the lumina of tracheids and fibres where fungal hyphae colonize, this assumption may be reasonable. Activities of the same type and amount of ligninolytic enzymes and free radicals produced by *P. sanguineus* may be restricted/or delayed (i.e. via a reduction in porosity) in $TMW_{220°C}$ due to increased lignin content and condensation. In our previous study on ash reference wood, compared to fibres, *P. sanguineus* produce narrow delignified zones in vessel and parenchyma cell walls that contain higher lignin and have differences in the syringyl/guaiacyl ratio compared to fibres (Kim *et al.*, 2015a). The second explanation is delay of delignification by modification of

cell wall structure. In our previous study, ash $TMW_{220°C}$ showed severe changes in the supramolecular structure of fibre S_2 layer (Kim *et al.*, 2015b). The lamella structure of the fibre S_2 layer was disrupted in $TMW_{220°C}$ followed by formation of larger lignin aggregates than reference (Kim *et al.*, 2015b). Several studies have also reported blocking of micropores and reduction in void volume in the wood cell wall following TM (reviewed by Esteves & Pereira, 2009). These observations suggest that the modified cell wall structure of $TMW_{220°C}$ can delay penetration/or diffusion of ligninolytic enzymes (i.e. laccase) and free radicals in the cell wall, thereby delaying delignification in $TMW_{220°C}$. Narrower transition zones in spruce tracheids and ash fibres of $TMW_{220°C}$ observed in this study may particularly reflect this aspect.

6 Soft rot decay patterns of Termovuoto treated hardwoods

To understand the effect of thermal modification (TM) on soft rot decay of TMW, samples (ash, beech) degraded by *P. mutabilis* for 18 weeks were examined using light microscopy combined with two histochemical staining approaches (i.e. toluidine blue and safranin staining). To examine ultrastructural changes in decay patterns of TMWs, TEM was carried out on ash $TMW_{200°C}$ decayed by *P. mutabilis* in comparison with decayed ash reference wood.

6.1 Histochemical observations of *P. mutabilis* decayed ash and beech TMWs

After 18 weeks exposure, hardwood (ash, beech) reference and TMWs showed typical pattern of cavity formation (i.e. soft rot Type-I) in fibre cell walls. Soft rot erosion (i.e. Type-II) attack by luminal hyphae was not observed in both reference and TMWs. The higher the treatment temperature, the less advanced decay stages detected in fibres. Ash and beech reference, ash $TMW_{190°C}$ and beech $TMW_{190–200°C}$ showed advanced stages of decay in which the entire secondary S_2 layer was completely degraded, leaving a residual S_3 layer adjacent to the cell lumina (Figure 17a, b, e−g; Paper IV) and undegraded middle lamella (ML) regions. For ash $TMW_{200°C}$ and beech $TMW_{210°C}$, decay morphologies differed slightly from those exhibited in the reference. The secondary cell walls contained large numbers of uniting cavities close to the fibre lumina, leaving only remnants of the outer secondary wall (Figure 17c, h). Ash $TMW_{210°C}$ showed only a few cavities of smaller diameter even though the cell lumina were filled with numerous hyphae (Figure 17d). For ash and beech $TMW_{220°C}$, cavity formation was almost completely inhibited and only very thin hyphae were found to propagate across wood cells (Figure 17i).

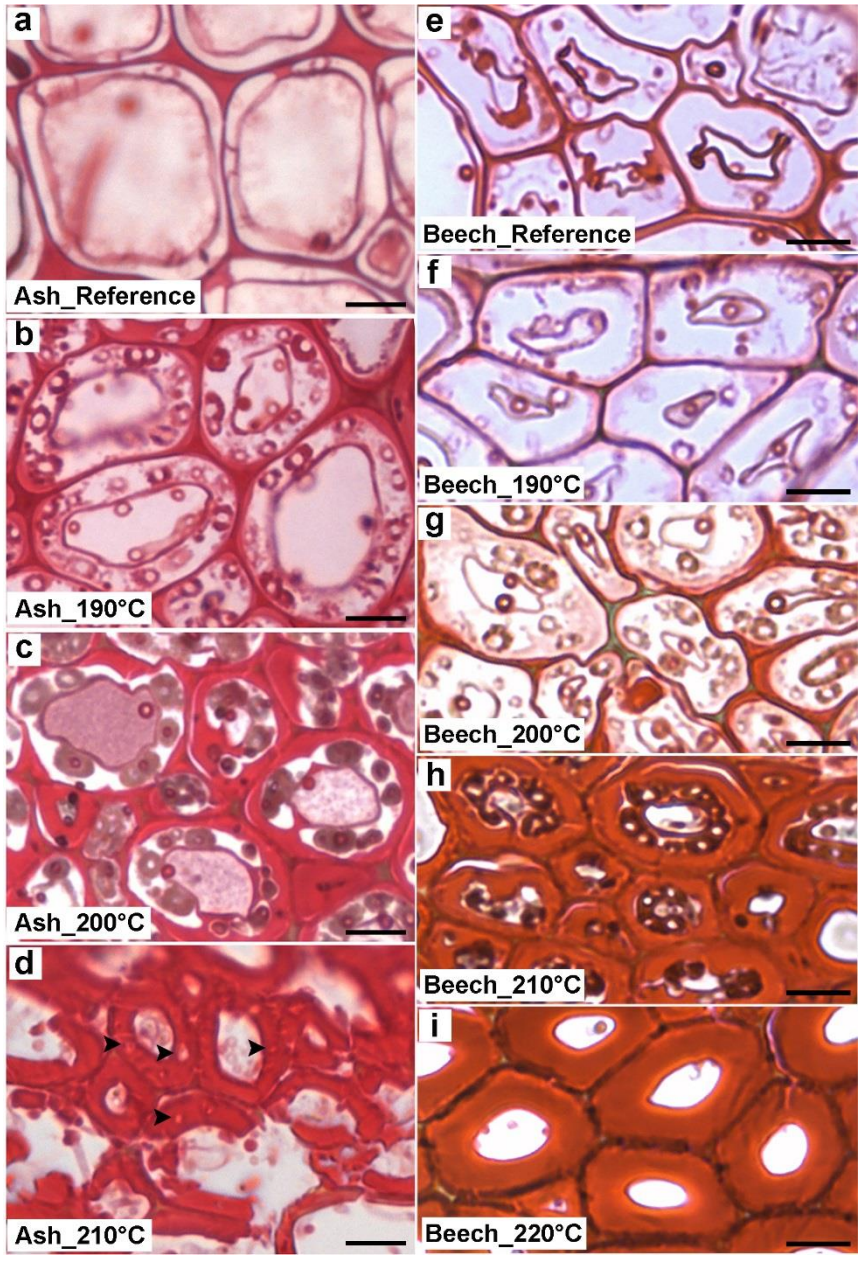

Figure 17. P. mutabilis decayed fibres in ash and beech reference and TMWs. Formation of typical soft rot Type-I cavities in the S_2 layer was shown in two references (a, e), ash $TMW_{190°C}$ (b) and beech $TMW_{190–200°C}$ (f, g), with a residual S_3 layer and undecayed ML regions. Ash $TMW_{200°C}$ (c) and beech $TMW_{210°C}$ (h) showed coalesced cavities restricted to inner regions of the S_2 layer. Ash $TMW_{210°C}$ (d) revealed only a few cavities (arrowheads) in the S_2 layer even though the colonization of numerous hyphae was observed in cell lumina. Beech $TMW_{220°C}$ (i) showed a few hyphae in cell lumina and no cavities in the S_2 layer. Scale bars = 20 μm.

Therefore, it is likely that an effect of TM on soft rot decay is to suspend cavity formation and thereby to delay/inhibit the decay process. The relative increased lignin concentration (i.e. about 10% higher in ash and beech $TMW_{210–220°C}$ compared to reference) due to TM is most likely the major factor influencing the resistance of wood (i.e. secondary cell walls) to soft rot decay (Section 4, Paper III). Higher natural resistance of ML regions in both reference and TMWs further reflect this aspect, i.e. the high lignin content in ML regions inhibits soft rot decay in these regions (Daniel, 2014). Possibly, the loss and/or redistribution of hemicelluloses and cellulose microfibrils and condensation of lignin following TM can also interfere with the fungus ability to produce T- and L-branching which is a prerequisite for subsequent cavity formation.

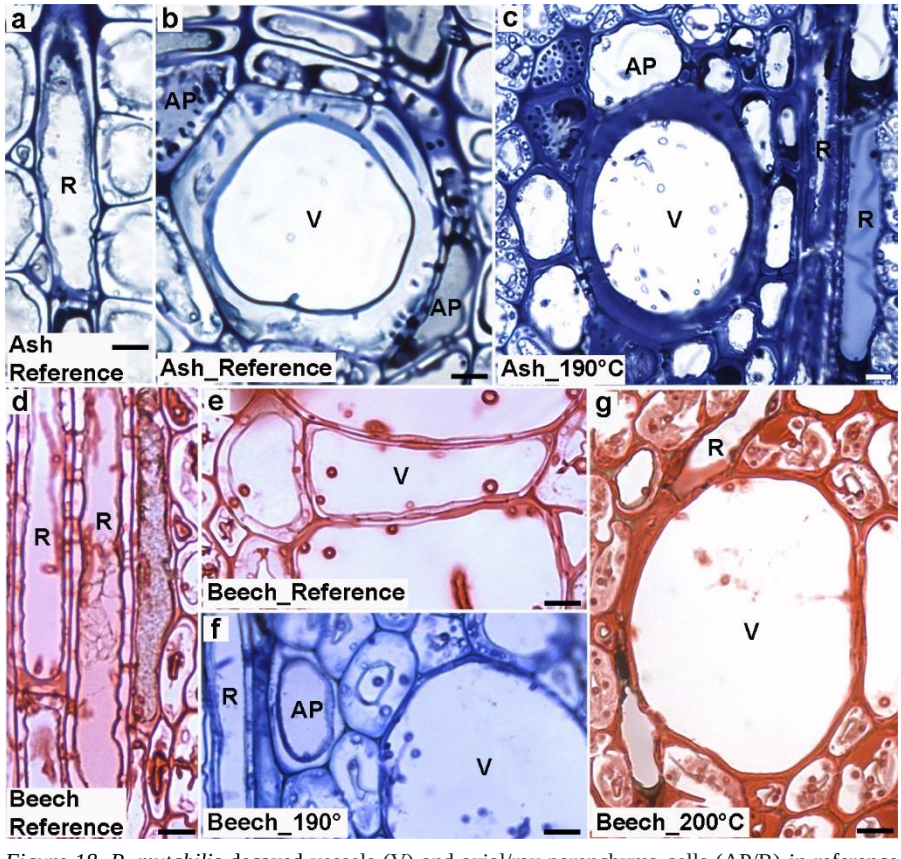

Figure 18. P. mutabilis decayed vessels (V) and axial/ray parenchyma cells (AP/R) in reference and TMWs. Stained with toluidine blue (a−c, f) and safranin O (d, e, g). Ash (a, b) and beech (d, e) references and beech $TMW_{190°C}$ (f) showed advanced stages of Type-I cavity formation in secondary cell walls, while no obvious cavity formation and decay were detected in ash $TMW_{190°C}$ (c) and beech $TMW_{200°C}$ (g). Scale bars =20 µm

In vessels and axial/ray parenchyma cells, ash and beech reference showed formation of soft rot type-I cavities in secondary cell walls (Figure 18a, b, d, e). Beech $TMW_{190°C}$ also exhibited similar advanced stages of decay in these cell types, with the secondary cell walls almost completely degraded (Figure 18f). However, no cavity formation was observed in vessels or parenchyma cells of ash $TMW_{190°C}$ (Figure 18c). Similar observations were seen in the case of beech $TMW_{200°C}$ (Figure 18g). Also no obvious cavity formation was detected in TMWs treated at higher temperatures in the two hardwoods. Consequently, the Termovuoto process significantly increased durability of vessels and parenchyma cells against *P. mutabilis* at 190/200℃ for ash/beech, respectively. These results differed from fibres that displayed notable increase in durability when the treatment temperature was raised to 210/220℃ (i.e. much higher temperatures than vessels and parenchyma cells). Thus, a considerable difference in soft rot decay resistance between vessels/parenchyma cells and fibres was evident. These differences in durability are probably originating from difference in the native chemical composition between cell types, for example, the proportion of lignin in vessels/parenchyma cells is higher than in fibres (Obst, 1982). Our previous research on ash reference also showed an overall stronger lignin staining in vessels/parenchyma cells than fibres (Kim *et al.*, 2015b). Furthermore, vessels and parenchyma cells generally contain tyloses and deposits of aromatic compounds and extractives, respectively. All these differences in chemical composition may contribute to a higher decay resistance in vessels/parenchyma cells than fibres in TMWs.

6.2 TEM observations of *P. mutabilis* decayed ash $TMW_{200°C}$

Since the mass loss results and light micrographs from decay tests suggest significant structural and chemical changes in ash wood during treatment temperatures around and above 200℃, characteristic decay patterns in ash $TMW_{200°C}$ by *P. mutabilis* was compared with ash reference wood using TEM.

Figure 19 shows advanced stages of cavity formation in fibres of ash reference and $TMW_{200°C}$, in which almost the entire S_2 fibre cell wall was destroyed through cavity formation. Distinct granular layers were formed around fungal hyphae (i.e. inside the secondary cell wall or in the cell lumina) consisting of electron dense materials as observed in both reference and $TMW_{200°C}$, with differences in the morphology, texture and amount remaining (Figure 19). The granular electron dense materials were retained after cavity formation and even after hyphal death, suggesting they presumably represent un-degraded/or modified lignin and fungal melanin, which react strongly with osmium tetroxide.

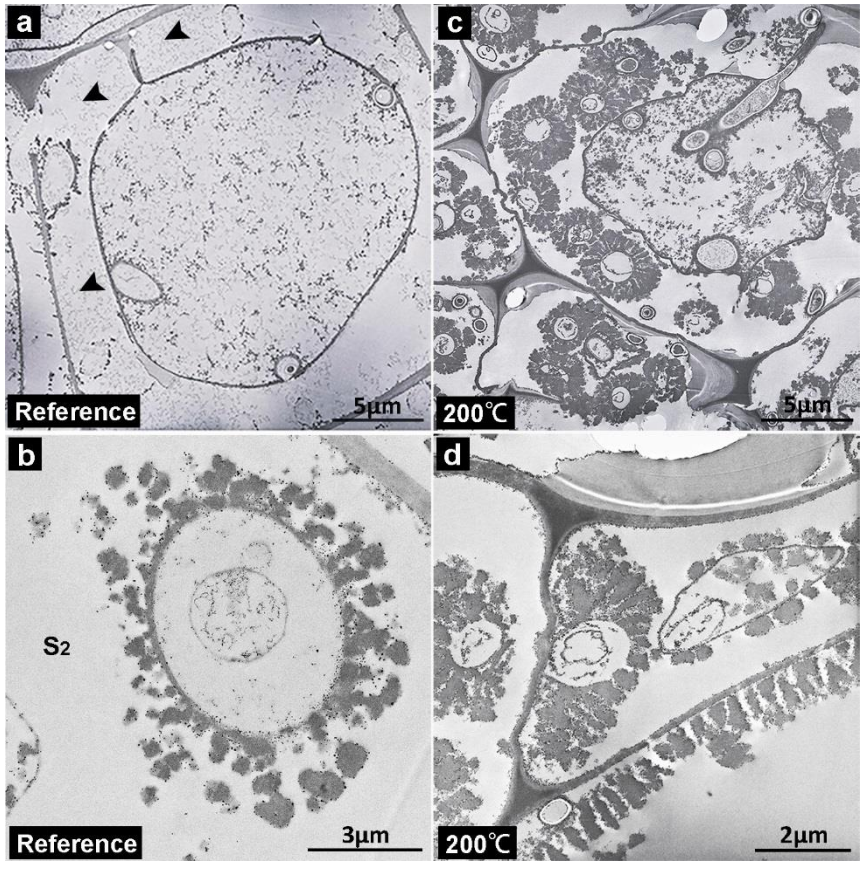

Figure 19. TEM micrographs of fibres of ash reference and $TMW_{200°C}$ decayed by *P. mutabilis*. Reference (a, b) and $TMW_{200°C}$ (c, d) showed advanced stages of decay; complete degradation of the entire S_2 layer, leaving a residual S_3 layer adjacent to the cell lumina and ML regions and deposition of electron dense materials and osmium particles around hyphae. Note a characteristic radial-like distribution of electron dense granular materials around hyphae located in the S_2 layer and close to the S_3 layer and ML regions of $TMW_{200°C}$ and lack of fibrillar-like materials in decayed $TMW_{200°C}$ fibre cell walls (c, d), which differed from reference (a, b).

Micromorphologically, the electron dense materials in the fibre S_2 layer showed different distributional patterns between reference and $TMW_{200°C}$. They formed a radial-like arrangement around hyphae in $TMW_{200°C}$ with amorphous granular material apparently expanding from hyphae (Figure 19c, d) differing from the more granular and open patterns observed in the reference (Figure 19b). This arrangement was also occasionally located close to the S_3 layer and ML regions of $TMW_{200°C}$ (Figure 19d). In decayed fibre cell walls, reference frequently showed abundant and widely distributed fibrillar-like materials (Figure 19a, arrowheads), while these materials were not observed in $TMW_{200°C}$ (Figure 19c, d). Variations in the distribution of osmium particles

and differences in the size of electron dense granules in decayed fibre cell walls between $TMW_{200°C}$ and reference were also noted.

These characteristic differences in degraded fibre cell walls between reference and $TMW_{200°C}$ presumably reflect changes in the decay mechanism by soft rot fungi which is most likely related to the modified cell wall structure and lignin chemistry following TM, such as polycondensation of lignin and formation of large lignin aggregates followed by loss of the lamellar orientation discussed in earlier sections. The modified lignin may also be prone to aggregate and polymerize with melanin secreted by the fungal hyphae and form an even more compact aggregate structure in the presence of osmium tetroxide. Consequently, the electron dense granular materials around cavities in fibres of $TMW_{200°C}$ were more apparent than those in the reference wood.

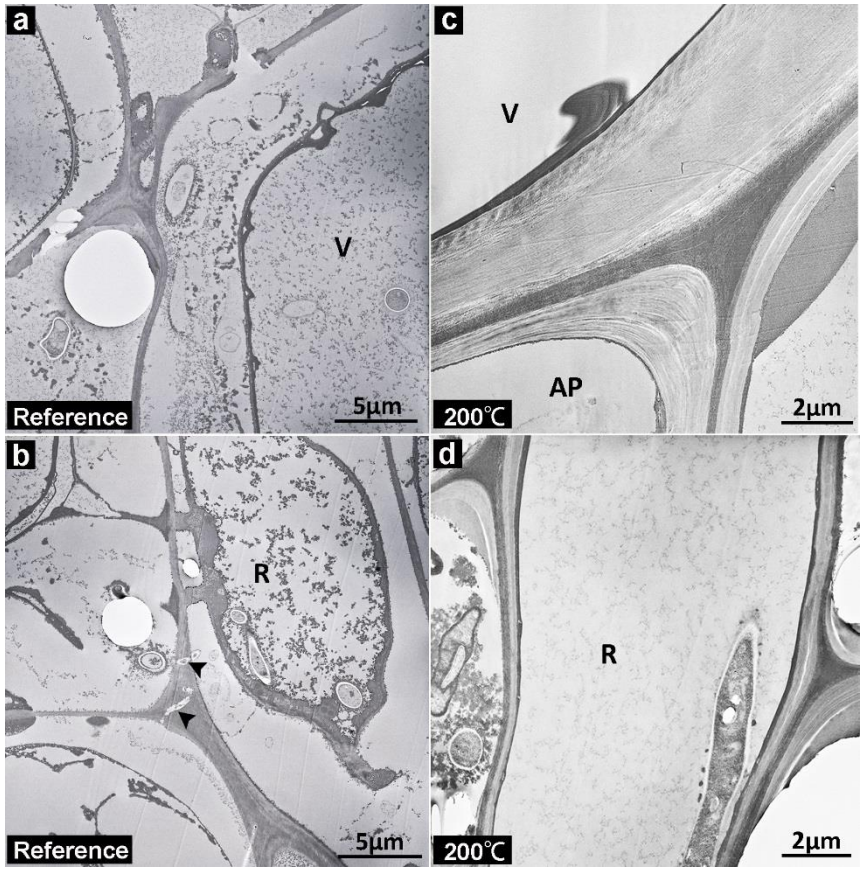

Figure 20. TEM micrographs of vessels (V) and axial/ray parenchyma cells (AP/R) of ash reference and $TMW_{200°C}$ decayed by *P. mutabilis*. Reference showed advanced stages of decay in secondary cell walls of vessels and parenchyma cells (a, b), while no obvious signs of decay were detected in $TMW_{200°C}$ (c, d). Note characteristic holes produced by micro-hyphae across ray parenchyma cell walls in reference (arrowheads in b), which were not observed in $TMW_{200°C}$.

In vessels and parenchyma cells, reference showed advanced stages of decay (Figure 20a, b), while no obvious decay was detected in $TMW_{200°C}$ even though neighboring fibres showed advance decay (Figure 20d). This indicates that durability of vessels and parenchyma cells was significantly improved following TM as outlined with light microscopy (Figure 18). Unlike $TMW_{200°C}$, reference showed some representative holes (bore holes; arrowheads in Figure 20b) of various shapes across vessel and parenchyma cell walls. These holes differed from characteristic cavities formed in the S_2 layer. Probably, these observations reflect the penetration of micro-hyphae produced from luminal hyphae. Consequently, the differences between reference and $TMW_{200°C}$ observed indicate changes in the action of soft rot decay in vessels and parenchyma cells following TM. Like fibres, modified cell wall chemistry and structure of vessels and parenchyma cells by TM (see Paper II) may induce these differences.

7 Conclusions

The Termovuoto (thermo-vacuum) process is a relatively new industrial approach that combines an efficient vacuum drying process with thermal modification (TM). Two softwoods (spruce and fir) and two hardwoods (ash and beech) were thermally modified under industrial conditions by the Termovuoto process for 3−4 h at 160−220°C and used throughout this thesis.

Changes in ultrastructure and lignin chemistry of TMWs were investigated using light- and electron microscopy combined with histo/cytochemistry. Variations in changes in native cell color in TMWs were positively correlated with differences in lignin content between cell types and cell wall regions in reference wood. Histochemical staining showed increasing amounts of acidic groups in TMWs with different response to toluidine blue or safranin between secondary cell walls and CMLcc regions. The CMLcc regions of softwood $TMW_{220°C}$ (4 h) were composed almost entirely of modified lignin with increased amounts of acidic groups. With cytochemical staining for lignin, many electron dense particles were detected in the CMLcc regions of softwood TMWs, indicating early degradation/modification by Termovuoto treatment. In hardwoods, increased intensity of lignin staining and large dark lignin aggregates in the fibre S_2 layers were detected in $TMW_{220°C}$ (3 h). Hardwood $TMW_{220°C}$ differed significantly in ultrastructure of fibre cell walls compared to reference, including loss of the lamellar structure and size and ultrastructural distribution of lignin aggregates. Modification in CMLcc structure in ash $TMW_{220°C}$ was different from that of softwood $TMW_{220°C}$.

Durability of TMWs exposed to two brown rot (*P. placenta*, *G. trabeum*)-, two white rot (*P. radiata*, *P. sanguineus*)- and three soft rot (*C. globosum*, *P. malorum*, *P. mutabilis*) fungi were investigated using the soil-block test (AWPA E10-08). For brown- and white-rot fungi, considerable improvement in durability for soft- and hardwoods was only achieved when the TM temperature reached 220°C. TM temperatures below 200°C (i.e. $TMW_{160°C}$ for softwoods and $TMW_{190°C}$ for hardwoods) occasionally influenced decay

resistance negatively in case of some fungal species in both soft- and hardwoods. Hardwood TMWs demonstrated an overall higher decay resistance than softwood TMWs at high TM temperatures. The durability of ash TMWs was higher than beech TMWs, meaning the behavior of TM differed between ash (ring-porous hardwood) and beech (diffuse-porous hardwood). Comparison between results of soil block (AWPA E10-08) and agar block (EN 113) tests demonstrated that the influence of testing method in terms of determination of durability classes is of less importance. For soft rot fungi, softwood TMWs were more durable than hardwood TMWs, irrespective of fungal species tested. In hardwoods, ash exhibited less durability than beech in untreated reference, but greater durability in TMWs, suggesting differences in the effect of TM depending on variation in anatomy between the two hardwoods as shown from brown- and white-rot decay.

Decay patterns in TMWs exposed to the white rot fungus *P. sanguineus* and soft rot fungus *P. mutabilis* were evaluated at tissue and cellular levels by various microscopy techniques. *P. sanguineus* attacked soft- and hardwood TMWs showed similar decay patterns as untreated reference wood in terms of fungal colonization, variations in decay between cell types and preferential degradation of lignin. However, results demonstrate that the delignification process in tracheids and fibres by *P. sanguineus* is delayed in TMWs, particularly at high treatment temperatures. *P. mutabilis* decayed hardwood TMWs showed evidence for formation of typical soft rot Type-I cavities in fibres at lower temperatures (190−200℃) similar to those in reference fibres. However, cavity formation in fibre cell walls was inhibited and/or delayed in TMWs at higher temperatures between 210 and 220℃. With TEM, decayed ash $TMW_{200℃}$ showed a radial-like distribution of electron dense materials in cavities and lack of fibrillar-like materials within degraded fibre walls, features differing greatly from reference. Presumably, differences in decay patterns of white- and soft rot fungi between reference and TMWs are due to the modified cell wall structure and chemistry following thermal treatment.

This study provides detailed information regarding chemical and ultrastructural changes, durability and decay patterns of TMWs against brown-, white- and soft-rot fungi. Fungal durability of TMWs is highly dependent on soft- and hardwoods, wood species, treatment temperature and fungal species. With the knowledge gained, it is important to take chemical and anatomical characteristics of selected wood species into consideration to choose the optimal Termovuoto conditions to meet the requirements for specific future purposes.

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