Gry Alfredsen*, Carl Gunnar Fossdal, Nina E. Nagy, Jody Jellison and Barry Goodell

Furfurylated wood: impact on *Postia placenta* gene expression and oxalate crystal formation

DOI 10.1515/hf-2015-0203
Received September 25, 2015; accepted February 20, 2016; previously published online March 24, 2016

Abstract: Modified wood can provide protection against a range of wood deteriorating organisms. Several hypotheses have been put forward regarding the protection mechanisms against wood decaying fungi including fungal enzyme inefficiency due to non-recognition, lower micropore size, and insufficient wood moisture content. The aim of this study was to obtain new insight into the protection manner of furfuryl alcohol (FA) modified Scots pine sapwood (W_{FA}), and to examine biochemical mechanisms and adaptive changes in gene expression utilised by *Postia placenta* during early colonisation of W_{FA}. Samples were harvested after 2, 4, and 8 weeks of incubation. After 8 weeks, the mass loss (0.1%) and wood moisture content (21.0%) was lower inW_{FA}, than in non-modified Scots pine sapwood samples (W), 26.1% and 46.1%, respectively. Microscopy revealed needle-shaped calcium oxalate crystals, at all harvesting points, most prominently present after 4 and 8 weeks, and only in the W_{FA} samples. Among the findings based on gene profiles were indications of a possible shift toward increased expression, or at least no down regulation, of genes related to oxidative metabolism and concomitant reduction of several genes related to the breakdown of polysaccharides in W_{FA} compared to W.

Keywords: brown-rot colonisation, furfurylated wood, gene expression, microscopy, oxalic acid, *Postia placenta*, quantitative real time PCR

***Corresponding author: Gry Alfredsen,** Department of Wood Technology, Division of Forest and Forest Resources, Norwegian Institute of Bioeconomy Research, Box 115, NO-1431 Ås, Norway, e-mail: gry.alfredsen@nibio.no
Carl Gunnar Fossdal and Nina E. Nagy: Department of Forest Health, Division of Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research, Box 115, NO-1431 Ås, Norway
Jody Jellison: Department of Biology, Center for Agriculture, Food and the Environment, 316 Stockbridge Hall, University of Massachusetts, Amherst, MA 01003, USA
Barry Goodell: Department of Sustainable Biomaterials, Virginia Tech, Blacksburg, VA 24060, USA

Introduction

Wood modification involves the action of a chemical, biological or physical agent upon the material, resulting in a desired property enhancement of the material (Hill 2006). The modified wood should itself be non-toxic under service conditions and there should be no release of any toxic substances at the end of service life. Modified wood can provide protection against wood deteriorating organisms, including decay fungi (e.g. Hill 2006, 2009; Lande 2008; Lande et al. 2008; Esteves and Pereira 2009; Rowell et al. 2009a,b; Papadopoulos 2010; Verma and Mai 2010). The science and technology of the different wood modification processes has been comprehensively described (Hill 2006, 2011).

Fungal action in wood treated with traditional broad-spectrum chemical preservatives is assumed to be different than in modified wood due to the toxic vs. non-toxic nature of the wood protection systems. An understanding of the mechanisms utilised by decay fungi to attempt to degrade modified wood is important for further optimisation of future modified wood products and for an expanded understanding of fungal decay in general. Ringman et al. (2014a) summarised in detail the hypothesis that have been put forward regarding the mode of action of modified wood and decay fungi. Briefly, the proposed mode of action principles include: (1) unavailability of easily accessible nutrients (Boonstra et al. 2007; Rowell et al. 2009a), (2) enzyme non-recognition (Rowell 2005), (3) micropore blocking (Hill et al. 2005), (4) moisture exclusion due to OH-group blocking/reduction (Rowell et al. 2009a) and/or reduction in void volume (Boonstra and Tjeerdsma 2006; Hill 2009).

The wood modification process called furfurylation involves an impregnation step with furfuryl alcohol (FA) and a curing step to polymerise FA. FA is often obtained from agricultural waste such as sugar cane, corn cobs, sunflower or birch chips (Maciel et al. 1982; Chuang et al. 1984; González et al. 1992, 2002; Lande et al. 2008). According to Lande et al. (2004), furfurylation of wood provides protection against bio-degradation (fungi, bacteria and marine borers) without reliance on biocidal treatments.

A review of the degradation of cellulose by basidiomycetous fungi is provided by Baldrian and Valaskova (2008), and brown-rot decay mechanisms are more currently reviewed by Arantes et al. (2012) and Arantes and Goodell (2014). Brown-rot fungi have also a non-enzymatic system as a tool: the chelator-mediated Fenton system, that rapidly depolymerises the polysaccharides and lignin in early stages of biodegradation (Goodell et al. 1997a; Arantes et al. 2011; Eastwood et al. 2011).

As a part of their normal metabolism, fungi are known to secrete substantial quantities of organic acids. Oxalic acid (OA) may accumulate as oxalate (OX) salt crystals on the outside of basidiomycota hyphae. OX is an effective chelator of cations including Fe, and it has important implications regarding both weathering of soil and the decomposition of wood (Eriksson et al. 1990). Regarding the latter, OA is assumed to play an important role in incipient stage of brown-rot decay, both as an iron chelator and as a phase transfer agent (Arantes and Goodell 2014).

Postia placenta is a common fungus in laboratory wood decay tests, e.g. the European standard EN 113 (CEN 1996a) and the American E10 (AWPA 2001). Martinez et al. (2009) have examined the *P. placenta* genome, transcriptome, and secretome revealing its extracellular enzyme systems. This included an unusual repertoire of extracellular glycosidases and the authors identified a large number of gene models supporting the activity of Fenton chemistry. Among the glycosyl hydrolases there are members of the glucose-methanol-choline oxidoreductase family (GMC oxidoreductase) with substantial similarity to known alcohol oxidases, aryl-alcohol oxidases, and glucose oxidases. Another gene potentially involved in Fe^{2+} production encodes a putative quinone reductase, which also would potentially be involved in the reduction of iron binding catecholates after oxidation to the quinone state in the redox cycling required in chelator-mediated Fenton reactions (Goodell 2003; Arantes et al. 2011). However, many of the fungal quinone reductases are membrane bound or intracellular, and therefore, there may be significant spatial issues that need to be considered. The *P. placenta* genome featured 236 P450 genes (Martinez et al. 2009). The P450s have various roles in the secondary metabolism. They are believed to be involved in the biodegradation of various xenobiotic compounds and lignin breakdown products. Extracellular accumulation of OX produced by *P. placenta* will: affect ferric iron availability, have a pH effect on a wide range of enzymes and also the phase transfer of iron from OX to catecholate/phenolate chelators. The later will sequester iron from OX at a pH generally above 4.2, and reduce iron to the ferrous form once the chelated-iron complex is within the wood cell wall. Hydroxyl radical formation occurs in that location rather than near the fungal hyphae, which prevents damage to the fungus by this potent radical species (Goodell et al. 2007; Arantes et al. 2012). Brown-rot fungi do not metabolise lignin, but they depolymerise and modify lignin extensively and the products remain *in situ* as a polymeric residue (Niemenmaa et al. 2007; Yelle et al. 2008). Martinez et al. (2009) did not find genes encoding exocellobiohydrolases and cellulose-binding domains *P. placenta*, but they did find an atypical brown-rot feature, i.e. that the *P. placenta* genome encodes two putative laccases.

In order to better understand the mechanisms of genes involved in the attack of wood cell walls, Vanden Wymelenberg et al. (2010) performed secretome and transcriptome analyses. As a carbon source they used ball-milled aspen or glucose. A range of hemicellulases but few potential cellulases were found to be produced by *P. placenta* and the expression patterns for oxidoreductase-encoding *P. placenta* genes were found to be consistent with an extracellular Fenton system. The white rot fungi *Phanerochaete chrysosporium* were found to produce a range of extracellular glycosyl hydrolases. Vanden Wymelenberg et al. (2011) examined gene expression of the same two fungi colonising aspen and pine. They concluded that the gene expressions of both fungi were influenced by the wood species and that the carbon source adaptions could reflect differences in decay mechanisms.

Tang et al. (2013) studied gene expression of the copper tolerant brown-rot wood decay fungus *Fibroporia radiculosa* at two time points. Southern yellow pine was treated with micronized copper quaternary compound (MCQ). When MCQ was protecting the wood, 58 genes were more highly expressed that had "putative functions related to oxalate production/degradation, laccase activity, quinone biosynthesis, pectin degradation, ATP production, cytochrome P450 activity, signal transduction, and transcriptional regulation". The authors identified several genes that seemed to be co-regulated, potentially linked to copper tolerance and/or wood decay. Kang et al. (2009) studied gene expression of selected decay enzymes (lignin peroxidase, manganese peroxidase and laccase) produced during biodeterioration of three wood types [pine, cedar, and alkaline copper quaternary (ACQ) treated pine] by the white rot fungus *Phlebia radiata*. They found that compared to pine and ACQ-treated pine the cedar reduced the microbial colonisation and its activities. Large amounts of laccase were produced by the fungus in ACQ treated wood but it was not able to cause any strength loss. Hence, it was concluded that the ACQ treatment was able to inhibit the effectiveness of the enzymes in some manner and that different resistant woods effected microbial colonisation

and enzyme activity differently during decay. Hietala et al. (2014) found that the suppressive effect of suboptimal temperature on *P. placenta* decay appeared more pronounced in Scots pine heartwood with increased durability compared to Scots pine heartwood with low decay resistance. This was particularly pronounced for cultures incubated at 30°C. Unlike sapwood, heartwood showed no mass loss, poor substrate colonisation and marker gene transcript level profiles indicating a starvation situation.

Molecular studies on the fungal decay of modified wood are so far rather rare. Pilgård et al. (2010) investigated fungal colonisation in various modified *Pinus sylvestris* sapwood by quantifying the fungal DNA of the white-rot fungus *Trametes versicolor* during 8 weeks of incubation. For all wood modifications, including furfurylated wood (W_{FA}), the maximum fungal biomass level was recorded after 2 weeks incubation. Pilgård et al. (2011) compared field stakes, including furfurylated *P. sylvestris* sapwood, after 6 years exposure in ground contact. They concluded that quantitative real-time PCR (qRT-PCR) in combination with microscopy provided relevant data about basidiomycete colonisation in wooden material and that qRT-PCR was a more sensitive tool than ergosterol and chitin analysis. Ringman et al. (2014b) found that the *P. placenta* genes presumed to be involved in oxidative depolymerisation (quinone reductase and alcohol oxidase) were expressed at higher levels in acetylated, DMDHEU-treated and thermally modified wood than in untreated wood. Although these enzymes are also suggested to be involved in other fungal metabolic activities (e.g. microbial detoxification reactions), it is intriguing that they were upregulated in the treated woods. For the genes investigated in enzymatic depolymerisation (endoglucanase and β-glucosidase), the levels of expression in modified woods were equal to or lower than those in untreated wood.

The referenced studies provided information that enhance our understanding of fungal biodegradation mechanisms. Expression of genes to produce enzymes and metabolites, not typically present during decay of untreated wood, might be activated in an attempt to adapt to growth in a stressed environment such as when faced with the challenge of obtaining nutrition and metabolising e.g. modified wood.

The aim of the present study was to obtain new insight into the manner of protection provided by the furfurylation of Scots pine and to reveal if there is a change in the biochemical mechanisms and gene expression by *P. placenta* during early colonisation of W_{FA} (2, 4, and 8 weeks). In particular this study focused on mass loss (ML) and wood moisture content (MC) in relation to: (1) gene expression as measured by qRT-PCR, (2) establishment of fungal colonisation profiles as observed by SEM, and X-ray microanalysis (EDXμA), and (3) chemical analyses of furfuryl alcohol and oxalate content in the wood.

Materials and methods

Wood samples and fungal inoculations: Mini-block samples, 5×10×30 mm^3 (Bravery 1979), were prepared from Scots pine (*P. sylvestris* L.) sapwood. The furfurylation process was performed on pre-cut mini-block samples with a 40% FA (Merck, Darmstadt, Germany) concentration on preconditioned samples (65% RH at 21°C until stable weight correspondig to 12% MC). The mean weight percent gain (WPG) in the FA samples (W_{FA}) was 14%. This is a lower level than typical commercial treatments and below the WPG 25 threshold indicated by Venås (2008). Low FA WPG was preferred to provide detectable decay within 8 weeks of exposure in order to provide comparison with the untreated wood (W) samples before these were too severely decayed. The authors are aware of that this comparison should be done cautiously. All samples were leached according to EN 84 (CEN 1996b). The samples were thereafter conditioned at 65% RH at 21°C for until stable weight was achieved. The samples were wrapped in sealed plastic bags before sterilisation by gamma irradiation of 25 kGY at the Norwegian Institute for Energy Technology.

Agar plates (TC Dish 100, standard, Sarstedt AG & Co., Nümbrecht, Germany) (Ø=87 mm, h=20 mm) containing 25 ml 4% (w/v) malt agar were inoculated with *P. placenta* (Fr.) M.J. Larsen & Lombard, strain FPRL 280. A plastic mesh was used to avoid direct contact between the samples and the medium. Two samples with the same treatment were incubated together in each Petri dish. The incubation time was 2, 4, and 8 weeks at 22°C and 70% RH with 12 samples (=six Petri dishes) prepared for each treatment at each harvesting point. After incubation, fungal mycelium was manually removed from the wood surface with Delicate Task Wipes (Kintech Science, UK); the samples were wrapped in aluminium foil and put directly into a container with liquid nitrogen. The samples were then stored at -80°C. Wood powder from frozen samples was obtained: the first 0.5 cm of the sample was removed (with an ethanol wiped saw), and then sawdust was produced by a sterile drill (Einhell SB 401/1, Landau/Isar, Germany) from the interior of the sample. Fine wood powder was produced in a Retsch 300 mill (Retsch GmbH, Haan, Germany). The wood samples, the 100-mg stainless steel beads (QIAGEN, Hilden, Germany) and the containers were chilled with liquid nitrogen before grinding at maximum speed for 1.5 min. Six biological replicates (one form each of the 6 Petri dishes) served for gene expression, four replicates (from four separate Petri dishes) for mass loss (ML) calculation and two for microscopy (from two separate Petri dishes). The oxalate (OX) and furfuryl contents were determined on the spare material from the gene expression replicates.

SEM and EDXμA: From the mini-blocks 10 mm long sub sections were prepared (with an ethanol wiped saw), and stored at -80°C until further processing. One block at the end and one in the middle of the mini-block sample were chosen for microscopy. Before examination, the samples were air dried for 12 h at room temperature (r.t.), a thin slice removed (with a razor blade) from the exterior radial surface, and wood material interior to that slice surface smoothed with a steel knife on a cryo-microtome (Microm HM 560 MV, Microm International GmbH, Walldorf, Germany). A radial orientation permitted

sectioning of the sometimes brittle wood and provided a smooth surface with less damage, as well as permitted evaluation of the colonisation status of wood material when viewed at lower magnification. For conventional SEM, the wood blocks were mounted on aluminium stubs (Senk Nagle 6x12 DIN 661 ALU, Tingstad as, Ålesund, Norway), sputter coated (Polaron Sputter Coater 7640, Quorum Technologies Ltd., East Sussex, UK) twice with gold-palladium, and viewed with a Zeiss EVO-50-EP SEM (Carl Zeiss SMT Ltd, Cambridge, UK).

For EDXμA of elements, the wood blocks were mounted by a carbon double-faced sticky tape (Pelco Tabs, 12 mm, OD, Ted Pella Inc., Redding, CA, USA) on aluminium stubs, air dried, and carbon coated (JEOL JEE-4X Vacuum Evaporator, JEOL, Peabody, MA, USA). Acquisition of digital images, in both secondary electron and backscatter mode, was performed via a 4Q-BSD (Four Quadrant Backscattered Detector type 603, K.E. Development Ltd, Cambridge, UK) and Link ISIS 300 X-ray analytical system (Oxford Instruments, Abingdon, UK).

Analysis of furfuryl (FA) content: Thermogravimetric analysis – TGA (PerkinElmer Thermogravimetric Analyzer, Pyris: (1) TGA, Pyris software 7.0-PerkinElmer, Wattham, MA, USA) was used for quantification of polymerised FA in the wood. van Riel et al. (2007) described a quality control system for W_{FA} by TGA. However, the method was modified here. The analysis started out with air 45 ml min^{-1}. When heating from 300°C to 600°C the air was changed to oxygen 45 ml min^{-1}. The temperature programme: (1) Initiate heating at 35°C for 1 min, (2) → 230°C with 30°C min^{-1}, (3) constant at 230°C for 45 min, (4) → 300°C with 20°C min^{-1}, (5) constant at 300°C for 50 min, (6) → 600°C with 40°C min^{-1}, (7) constant at 600°C for 6 min.

Similar to the analysis of van Riel et al. (2007), the improved model used a PLS regression model and was based on an initial set of 60 samples with a known variation in the FA content. The model was tested on a validation data set. The precision of the method turned out to fairly high, with a StD of 0.16 for the lowest level (WPG 18.8), 0.24 for the medium treatment level (WPG 27.2) and 0.25 for the highest level (WPG 42.9%). In the current study, both W_{FA} drill powder and W_{FA} fine powder were analysed by TGA, and the WPG was determined.

Analysis of OX: Wood material from the inoculation experiment was subjected to OX analysis according to Schilling and Jellison (2004). The samples were heated overnight at 105°C and the weight losses (WLs) were measured for the determination of the MC. Soluble and total OX analysis was performed on 50–100 mg samples of the ground material by extraction with either 1.5 ml distilled water or 1 M HCl (Sigma-Aldrich, St. Louis, MO, USA), respectively, as described by Rahman et al. (2007). The suspension was heated to 100°C in a heating block for 18 min. After cooling, the solution was centrifuged and filtered into new vials. In addition, the HCl-soluble OX was buffered with phosphate (Merk, Darmstadt, Germany) (0.111 M). Instrument: Agilent HPLC (Aligent Technologies, Santa Clara, CA, USA) with Diode Array Detector monitored at λ=210, with an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) 9 μm pore size, 300×7.8 mm I.D. column with a pH 1–3 sulfuric acid (Merk, Darmstadt, Germany) (20 mM, pH 1.40) mobile phase. The flow rate was 0.6 ml min^{-1} with a sample injection volume of 20 μl. The OX is well resolved from other components in the extracts, and accurate quantification of OX concentrations is possible in the range of 0.005–2 mM.

The pH was measured in W and W_{FA} exposed for 8 weeks of *P. placenta* decay with a PHM220 Lab pH meter and pHc 2401-8 combined pH electrode (Red rod) (Radiometer Analytical SAS, Villeurbanne Cedex, France).

Gene expression: The target genes and the endogenous controls in this study are listed in Table 1. The qRT-PCR gene specific primers used to determine the transcript levels were designed with Primer 3 software (Rozen and Skaletsky 2000) following the criteria: melting temperature 60°C and product size inferior to 120 bp. The target specificity of each primer set was examined by melting point analysis. Only primer pairs that gave a single melting peak and PCR product were applied. The RNA samples were DNAse treated with DNA-Free (Ambion®, Life Technologies, Foster City, CA, USA) prior to cDNA synthesis to avoid any amplification of genomic DNA. To control for a possible change in the target genes, three endogenous controls representing constitutively expressed housekeeping genes were used.

RNA was extracted from 100 mg aliquots of the pulverised wood samples by means of a Ribopure kit (Ambion®, Life Technologies, Foster City, CA, USA). DNA was removed from the samples via a DNA-free kit (Ambion®, Life Technologies, Foster City, CA, USA). RNA concentration was measured with a VersaFluor Fluorometer (BIO RAD, Hercules, CA, USA) and Quant-iTTM RiboGreen® RNA assay kit (Life Technologies, Foster City, CA, USA). Because RNA is unstable, cDNA was made using a TacMan® reverse Transcription system with Oligo d(T)16 (Applied Biosystems®, Life Technologies, Foster City, CA, USA) and amplified with PCR (GeneAmp PCR System 9700, Applied Biosystems, Foster City, Foster City, CA, USA). The genes analysed and their primer sequences are listed in Table 1.

Real-time PCR (7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) was performed on cDNA samples with 12.5 μl of 1× TacMan® SYBR®green mix (Applied Biosystems, Foster City, CA, USA) and 250 nM of each primer. The following qRT-PCR cycling parameters were used in the Absolute quantification mode: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s and annealing and replication at 60°C for 1 min, ending with a terminal melting curve determining step, where the temperature was increased linearly from 55 to 95°C for each PCR amplicon. A no-template control was run for each primer pair in each run to ensure that the primers did not form primer dimers or other PCR artefacts. All procedures above were performed according to the protocol provided by the manufacturers. All gene expressions (transcript levels) were calculated by means of the formula of Pfaffl (2001) with two fungal *α-tubulins* and one *β-tubulin* and as reference transcripts.

Statistics: A single-step multiple comparison procedure, Tukey's test (JMP, Version 10, SAS Institute Inc., Cary, NC, USA), was performed to compare means. A probability of ≤0.05 was used as a statistical type-I error level. Test of homogeneity of variance was done by both the O'Brien test and the Brown-Forsythe test. When the homogeneity of variance assumption was not fulfilled, this was indicated in the figure together with the respective p values. PLS analysis was performed in Unscrambler (Camo, Menlo Park, CA, USA). The analysis is based on an improved version of the model given in van Riel et al. (2007).

Results and discussion

Mass loss, wood moisture and FA content

The mean mass loss (ML) and wood MC after 2, 4, and 8 weeks of incubation are given in Table 2. In W_{FA} samples

Table 1: Target genes and endogenous controls, protein id, primer abbreviation and forward/reverse primers are listed. All *Postia placenta* sequences are from the Joint Genome Institute (JGI) database (http://genome.jgi-psf.org).

Gene	Protein ID	Primer abbreviation	Gene
Endogenous controls:			
β-tubulin	113871	βT-F/	CAGGATCTTGTCGCCGAGTAC/
		βT-R	CCTCATACTCGCCCTCCTCTT
α-tubulin	123093	αT1-F/	GCCCTACCGGTTTCAAGCTT/
		αT1-R	GCCAAGTCACCACCAGGAAT
α-tubulin	123093	αT2-F/	GGAGTCGCCTTGACCACAA/
		αT2-R	TGCCCTCACCAACGTACCA
Target genes:			
Oxidative depolymerisation			
Alcohol oxidase	118723	AlOx1-F/	CATCAAGAGCGCCAATCCAT/
		AlOx1-R	GGCGCAAAGTCAGCCTTGT
Chloroperoxidase	25391	ChlP-F/	CCGCCAGATGAACGCG/
		ChlP-R	AAGCAGAACGTGGGCGC
Copper radical oxidase	56703	CRO-F/	TGGCGGAACATACACGATCA/
		CRO-R	TGGCGGAACATACACGATCA
Laccase 1 Pplcc1	111314	Lac1-F/	CATCAATGGCACTGCAGAGC/
		Lac1-R	AGCCATTCCCAGGATTCAGA
Laccase 2 Pplcc2	89382	Lac2-F/	CGGTGCTCTTGGCCACTTAG/
		Lac2-R	CCATTGGTTATGGGCAGCTC
Monophenol oxidase	114245	MoPhe-F/	CCTCGCTTCATTCCCTGGAT/
		MoPhe-R	CCTCGCTTCATTCCCTGGAT
Mn peroxidase-like	44056	MPOX1-F/	GCATGCAGGTGGCAGTCTTT/
		MPOX1-R	CGAATCCTTGATTTGCCAGC
oxidoreductase	44654	Ox1-F/	TGAAGACCTTGTCCCTCCGA/
		Ox1-R	GCTGATGTGGATTGGTCCGT
NADH-quinone xidoreductase	124517	QRD-F/	CGACGACAAGCCCAACAAG/
		QRD-R	GATGACGATGGCGATTTTAGG
Enzymatic depolymerisation of holocellulose			
Endo-β-1,4-glucanase (GH5)	117690	GH5-F/	GTTCAGGCCGCATTGTCCT/
		GH5-R	TTCCACCTGGCGTAATTGTG
β-glucosidase (GH3)	107557	βGlu1-F/	CGGGTAACGCCATTGTTGAT/
		βGlu1-R	GCGCTTCGCAATGGTGTAC
Putative β-glucosidase (GH3)	112501	βGlu2-F/	TGCGCACGAATGAGTTGATAG/
		βGlu2-R	TGCGCACGAATGAGTTGATAG
Glucan 1,3β-glucosidase (GH55)	105490	GH55-F/	GGTCAGCGCTCAGGATCATT/
		GH55-R	TGGCGATGGCTGGTAGTATG
Glucoamylase (GH15)	117345	Gamy-F/	ATCACCGGGTCCGTTTCA/
		Gamy-R	TCGCGGAGGACATGATCA
Iron reduction and homeostasis			
Glycopeptide	128974	GlyP-F/	GATCTCCCCGCACTCGTT/
		GlyP-R	CCGGATGCGCCGTAGT
Phenylalanine ammonia lyase	112824	Pal-F/	CCGCGACGGGAAGATG/
		Pal-R	CGCCGCCGCAGAACT
Quinate transporter	44553	QuiT-F/	GAAGGGCCGCAGTCTTGAG/
		QuiT-R	GCGCTTCTCGGCCTTGA
Cytochrome P450 monooxygenases			
Cytochrome P450	110015	EP450-F/	AAGGCGTTCAACCCGTTCT/
		EP450-R	AGCTCCATGCTCGCCAAGT
Cytochrome P450	130132	P450 1-F/	GCCGTCCTTGACCACCTTT/
		P450 1-R	TCATCATCCTCGGCCAATG
Cytochrome P450	128850	P450D-F/	GCCTCATCCTAAAGCGCTACA/
		P450D-R	AAAGCGGTGAGCCGTCTGT
Oxalic acid			
Cytosolic oxaloacetase	112832	CyOx-F/	TGCACATCGAGGATCAGGTG/
		CyOx-R	AGACGACCTGCTTGCCCAT

Table 1 (continued)

Gene	Protein ID	Primer abbreviation	Gene
Glyoxylate dehydrogenase	121561	GlyD1-F/	CCTCACGTCGCACACAGTCTT/
		GlyD1-R	TGCCGCTGAGAGGTTGGT
Glyoxylate dehydrogenase	121565	GlyD2-F/	GAGATCGTCCCCGAGATGGT/
		GlyD2-R	TCAGTCGGAACGGAGACAATG
Formate dehydrogenase	98518	ForD-F/	CGAGGGTTCCCGCAACT/
		ForD-R	GAGCCAAGCACCCTTCTTGA
Oxaloacetate acetylhydrolase	112832	OxAH-F/	CTGCTTTCGGCGTGTGTTC/
		OxAH-R	CCATTTTGTATGTGATGAAGTCCTCTT

*NV=Ppl51393, Ppl116700, Ppl110711, Ppl115648, Ppl108962, Ppl103677, Ppl121251, Ppl128225, Ppl56055.
*NV, No measurable Cq value.

Table 2: Mean mass loss (ML) and wood moisture content (MC) (with StD) for W_{FA} and W after 2, 4, and 8 weeks of incubation with *P. placenta*. *n*=6.

	Incubation time (weeks)		
	2	4	8
ML (%)			
W_{FA}	0 (0)	0 (±1.3)	0.1 (±1.6)
W	2.7 (±0.5)	11.9 (±4.6)	26.1 (±2.6)
MC (%)			
W_{FA}	19.4 (±1.5)	19.6 (±2.0)	21.0 (±1.9)
W	27.3 (±1.1)	40.0 (±4.3)	46.1 (±3.0)

only 0.1% ML was recorded after 8 weeks. In the W samples, the MLs were much higher, i.e. 2.7, 11.9, and 26.1% after 2, 4, and 8 weeks, respectively.

The MC was much lower in all W_{FA} samples, reaching a mean of 21.0% after 8 weeks as compared to W samples, which reached a mean of 46.1% MC after 8 weeks. Hence, polymerised FA seems to be able to physically block, or delay, moisture access. This finding is in agreement with the well-established hypothesis that low MC is one of the factors explaining how W_{FA} resist degradation by decay fungi. Microscopy- and gene-expression data show, however, that MC was adequate to support fungal colonisation when nutrient media was applied to support the fungal growth. Under these conditions, enzymatic activity was also apparent. Fungal colonisation and decay however, depend upon free water in the cell lumen. Even if modified wood can lower the fibre saturation point (FSP) it will "not affect the amount of free water in the lumens at a given EMC" (Verma and Mai 2010). However, more knowledge is needed on the moisture distribution on cell wall scale in modified wood including the effects of moisture fluctuations and changes during different stages of decay.

The content of FA in W_{FA} material after 4 and 8 weeks of fungal decay was calculated as a change relative to the FA at 2 weeks of incubation. For drill powder and fine mill powder, the relative FA content (% based on total W_{FA}) increased with time. The FA increment was higher in drill powder (49.8% after 4 weeks, 72.0% after 8 weeks) than the fine powder (33.2% after 4 weeks, 58.8% after 8 weeks). The method for WPG quantification was not optimised to measure WPG change during brown-rot decay, but the results indicate that FA did not undergo significant degradation during the 8 weeks of incubation. The wood powder size influenced the TGA results.

SEM and X-ray microanalysis

Hyphae of *P. placenta* were observed in both W_{FA} and W samples (Figure 1). Hyphal production increased in all samples over time, from 2 to 4 to 8 weeks of incubation. More hyphae were found in regions of the longitudinal (axial) resin canals (Figure 1a and b) than in other cell types. Bosshard and Hug (1980) described the anastomoses of the resin canal system in three European conifers including *P. sylvestris*: "Intertwinements of axial ducts with rays initiate the formation of the radially oriented canal system". These cells and canals represent readily available routes for the fungus to access the wood.

In the W_{FA}, several types of hyphae with different morphological appearances were observed. Characteristically, the surfaces of the fungal hyphae were covered with crystals of different morphologies, varying from pointed, needle-shaped types, to more flattened, quadratic (square), and grainy shaped structures. Smooth hyphae dimpled with small round structures were also found. The needle-shaped crystals were detected at all harvesting points of incubation, and were increasingly present after 4 and 8 weeks of incubation, but only in the W_{FA} samples (Figure 1c, d and e).

In the W samples, hyphae of *P. placenta* were observed at all harvesting points. The surfaces of these hyphae were smooth and absent of any type of crystals. The hyphae

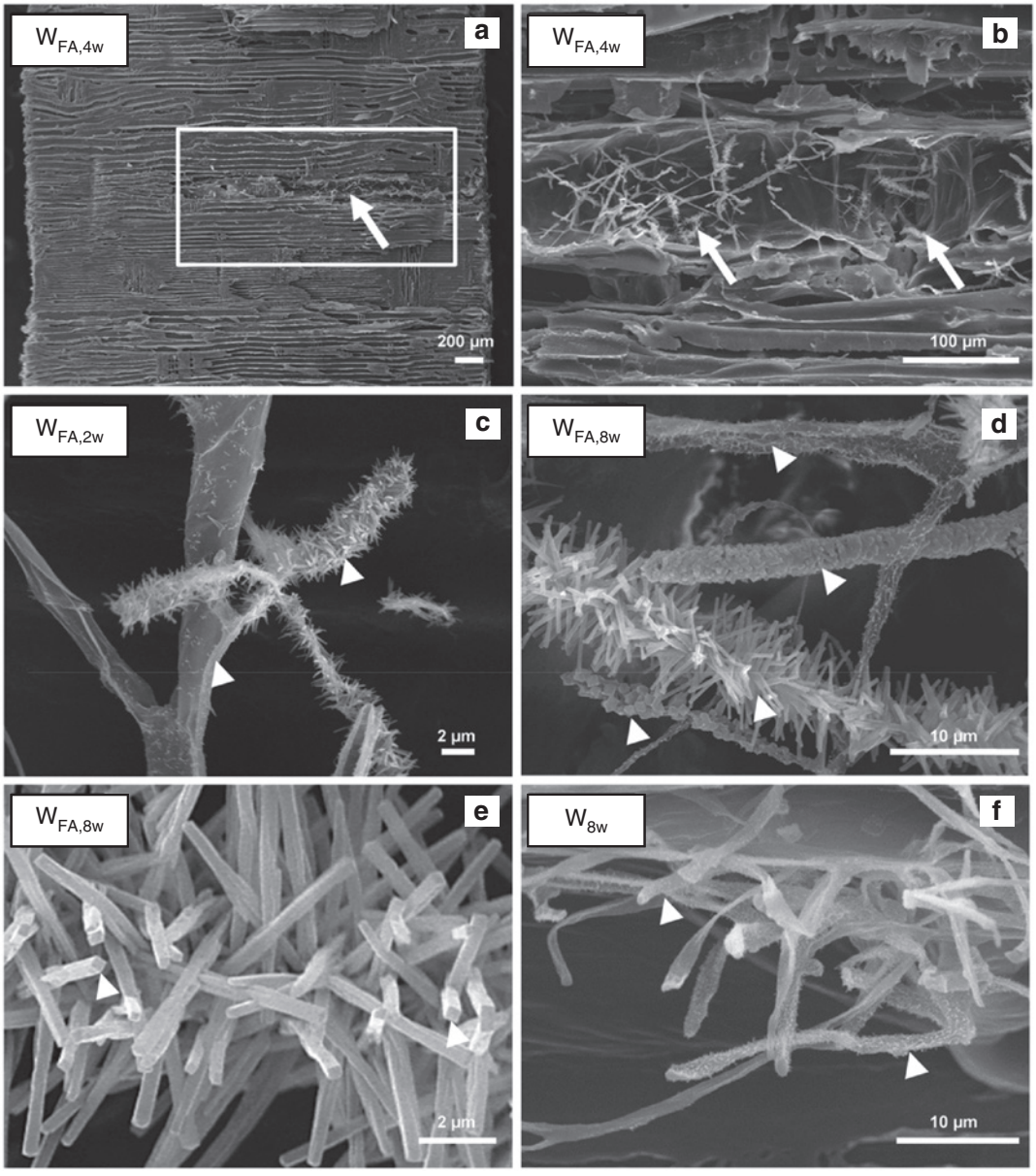

Figure 1: SEM images of colonisation of *P. placenta* in W_{FA} and W.
(a) Radial surface of $W_{FA,4w}$ wood block, a longitudinal resin duct is indicated. (b) A higher magnification from image A of the longitudinal resin duct with *P. placenta* hyphae aggregation. (c) Types of hyphae morphology in $W_{FA,2w}$. (d) Types of hyphae morphology in $W_{FA,8w}$. (e) Elongated crystals with square transverse section. (f) Hyphae in W_{8w} growing in between tracheid cells walls dimpled with small round structures.

were present either as single threads, or in bundle-like mycelial structures (Figure 1f). These hyphae were often covered with dot-shaped structures of similar appearance as seen in hyphae growing in W_{FA}.

EDXµA (Figure 2) revealed the presence of Ca in the crystalline structures, especially in the large crystals (spectrum 1), and less Ca was seen on hyphae with small crystals (spectrum 2) and no significant Ca was detected in the hyphae without crystals (spectrum 3). The production of Ca-OX crystals of varying morphologies has been observed for multiple fungal species and their presence correlated with multiple environmental factors including carbon excess, phosphorous levels, pH, etc. (Sayer et al. 1995; Jarosz-Wilkołazka and Gadd 2003).

Analysis of oxalic acid (OA) and oxalate (OX)

The data from the three different harvesting points indicate (Table 3) that in the W_{FA} samples, the total value for acid soluble OA and OX increased from 0.32 mg g^{-1} (week 2) to 0.53 mg g^{-1} (week 8), whereas for the W samples during

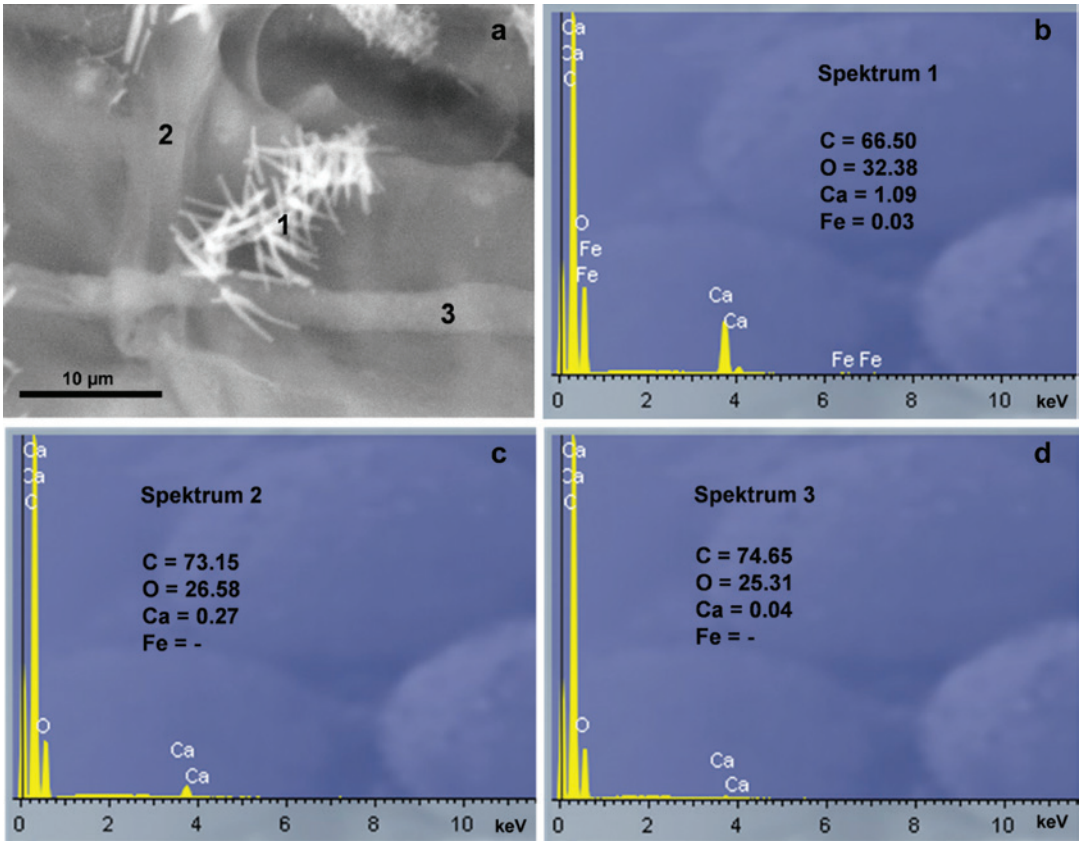

Figure 2: Precipitates on *P. placenta* hyphae in W_{FA} samples examined by EDXA.
(a) SEM in back scatter mode. Numbers 1, 2 and 3 indicate the areas corresponding spectra sites of each analysis. 1=hyphae with crystals; 2=smooth hyphae with initial crystals; 3=smooth hyphae without crystals. (b, c, d), Diagrams of EDXA spectrum 1, 2, and 3, respectively, annotated in figure (a) are consistent with oxalate crystal production.

Table 3: Production of soluble and insoluble oxalate (OX) by *P. placenta* in W_{FA} and W after 2, 4 and 8 weeks of incubation.

	Incubation time (weeks)		
	2	4	8
OX and acid soluble OA (mg/g)			
W_{FA}	0.319 (±0.017)	0.389 (±0.024)	0.533** (±0.083)
W	0.895 (±0.205)	0.539* (±0.168)	0.026** (±0.058)
Water soluble OA (mg/g)			
W_{FA}	0.270 (±0.040)	0.372* (±0.028)	0.499** (±0.089)
W	0.444 (±0.106)	0.259* (±0.083)	0.000** (±0.000)

Asterisks denote significant differences ($P \leq 0.05$) between the prior sampling period (*), or both prior sampling periods (**). There were always significantly different between W_{FA} and W except for week 4 for acid soluble OA and OX. n=6.

the same time period it was at 0.90 mg g^{-1} (2 weeks) and decreased to 0.03 mg g^{-1} (week 8). Water soluble OA followed a similar pattern, in the W_{FA} samples increasing from 0.27 to 0.50 mg g^{-1} during the study whereas in the W samples water soluble OA production was 0.44 mg g^{-1} (week 2) and decreased to zero (week 8). The last observation could also be explained by the fact that sugar was used in the malt agar. The OA and OX data show an OX increment in W_{FA} during incubation and a corresponding decrease in W, but the data cannot be explained by crystal formation in W_{FA} alone. Many fungi, both brown- and white rot fungi can produce Ca-OX crystals that adhere to the hyphae (Connolly and Jellison 1995) and are located within the fungal sheath. It has been suggested that Ca-OX precipitation along the hyphae of fungi can be an intracellular, periplasmic, or intramural nucleation with subsequent exteriorisation of the growing crystals (Connolly and Jellison 1995). The substrate pH modulation facilitates the activity of fungal extracellular enzymes that have pH optima below 5 (Punja et al. 1985). According to Connolly et al. (1996) “the study of crystal production patterns and crystal morphologies could yield important information about the microenvironmental conditions in wood during biodegradation and the mechanisms by which wood decay fungi decompose lignocelluloses”. Goodell et al. (1997b) demonstrated that when brown-rot fungi are

exposed to other non-biocidal treatments that increase pH and inhibit fungal growth on wood, copious quantities of water-insoluble OX crystals are produced on the fungal hyphae and on the surface of the adjacent substrate. The authors proposed that in a relatively high pH wood-cement medium (Goodell et al. 1997b) the fungi mount an aggressive effort to reduce the pH to levels where the chelator-mediated Fenton system could be active (Xu and Goodell 2001; Arantes et al. 2012). In the current study, the pH values in W_{FA} did not change significantly during the incubation period (pH 3.11±0.01 for non-decayed samples and 3.11±0.06 after 8 weeks). The pH of W was 4.77±0.03 in non-decayed samples and 3.19±0.13 after 8 weeks of incubation.

The shape of the rhomboidal shape of crystals identified in the W_{FA} (Figure 2) in combination with the EDX indicated the presence of Ca-OX crystals. The unique elongated clusters of long crystals with square cross-sections have not yet been described (to our knowledge) for *P. placenta* infected wood (Jarosz-Wilkołazka and Gadd 2003). The heavy production of this type of crystal can be interpreted as indirect evidence of the effort the fungi are expending to initiate pH change. However, the complete role of OX production in wood decay is not known exactly.

Gene expression

The three endogenous reference genes showed similar trends (data not shown) in expression with the strongest response caused by β-tubulin. Hence, this reference gene was considered best suited for statistical comparison and only *β-tubulin* results will be presented in the following. Figures 3–7 below shows the gene expression results for all target genes normalised against *β-tubulin*.

However, a direct comparison of the fungal gene expression in the two different materials is not

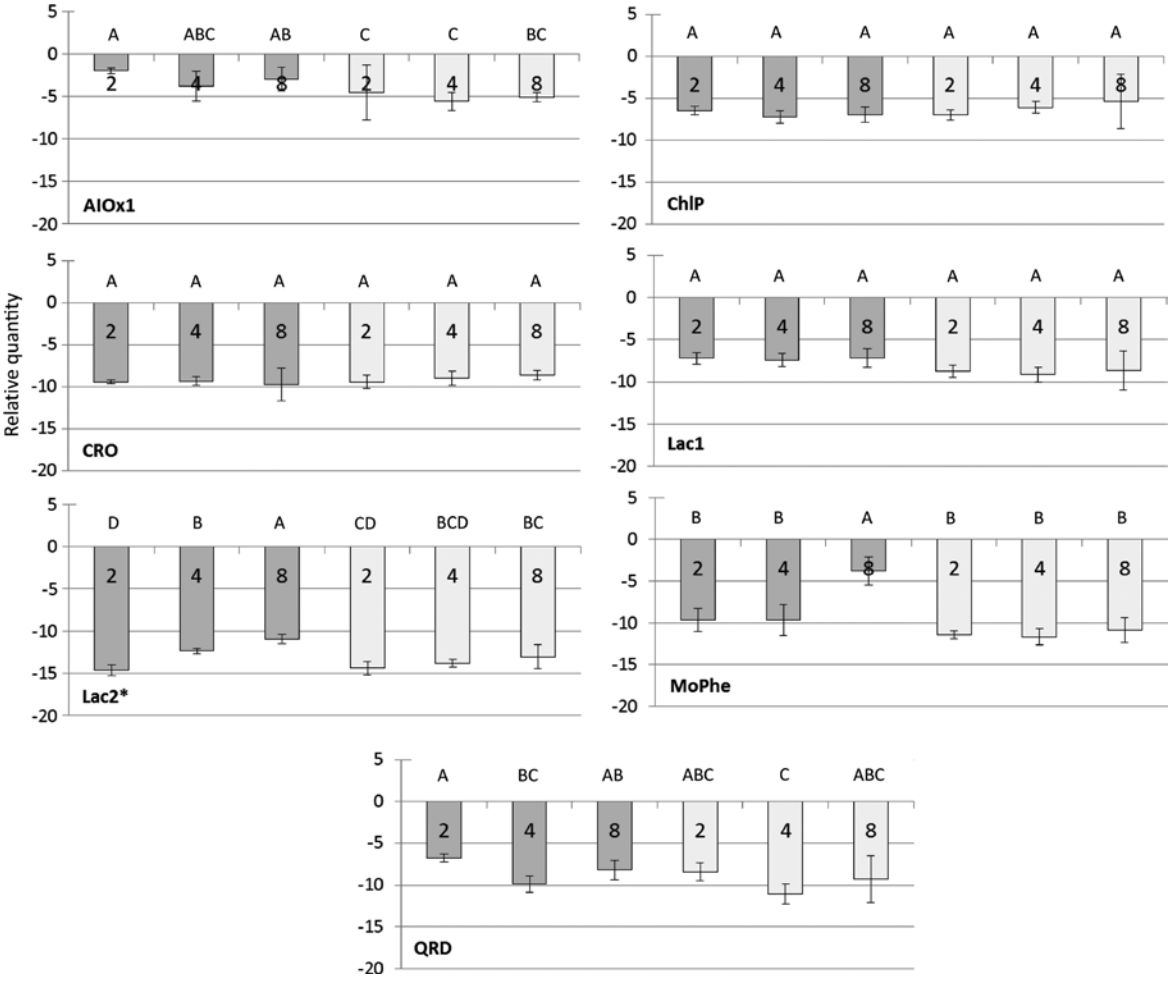

Figure 3: Genes involved in oxidative depolymerisation.
Mean and StD for tested target genes after 2, 4 and 8 weeks of incubation (n=6).
W_{FA} in dark grey (left), W in light grey (right). *Significant differences between the variances; O'Brian P=0.008, Brown-Forsythe P=0.0041.

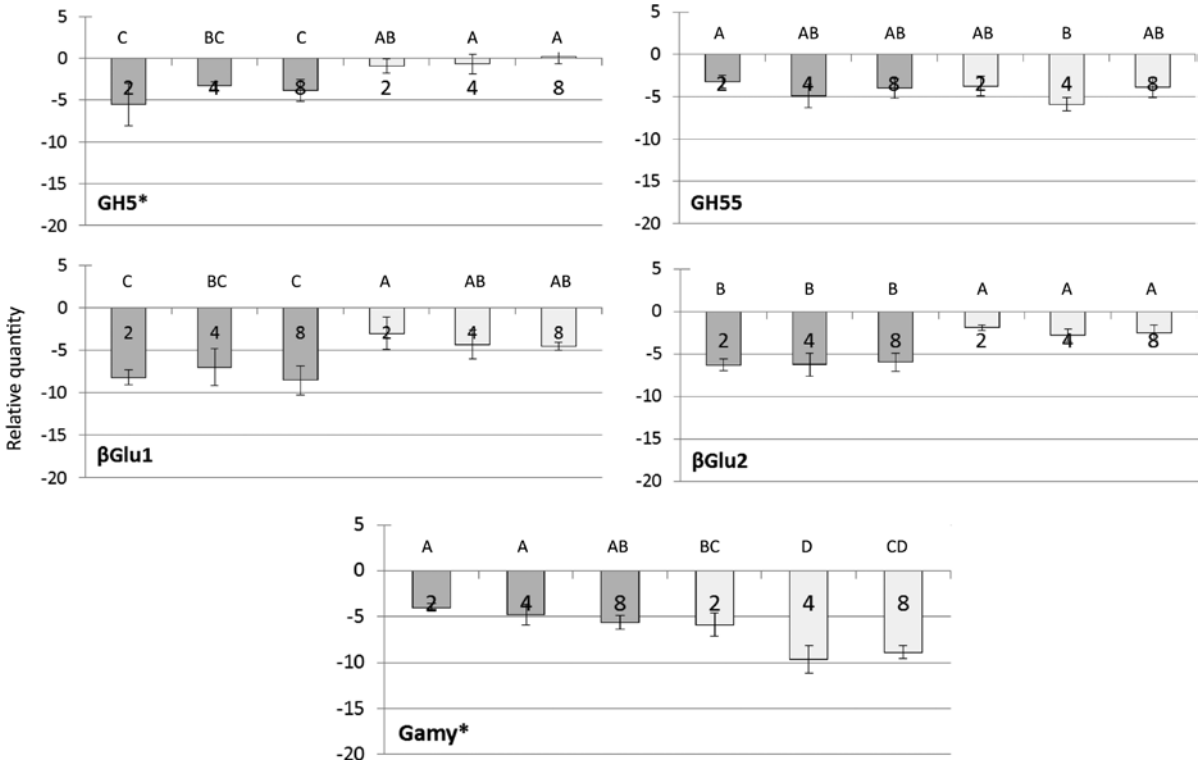

Figure 4: Genes involved in enzymatic degradation of polysaccharides.
Mean and StD for tested target genes after 2, 4 and 8 weeks of incubation (n=6). W_{FA} dark grey (left), W light grey (right). *Significant differences between the variances; *GH5*: O'Brian P=0.004, Brown-Forsythe P=0.02. *Gamy*: O'Brian P=0.04, Brown-Forsythe P=0.04.

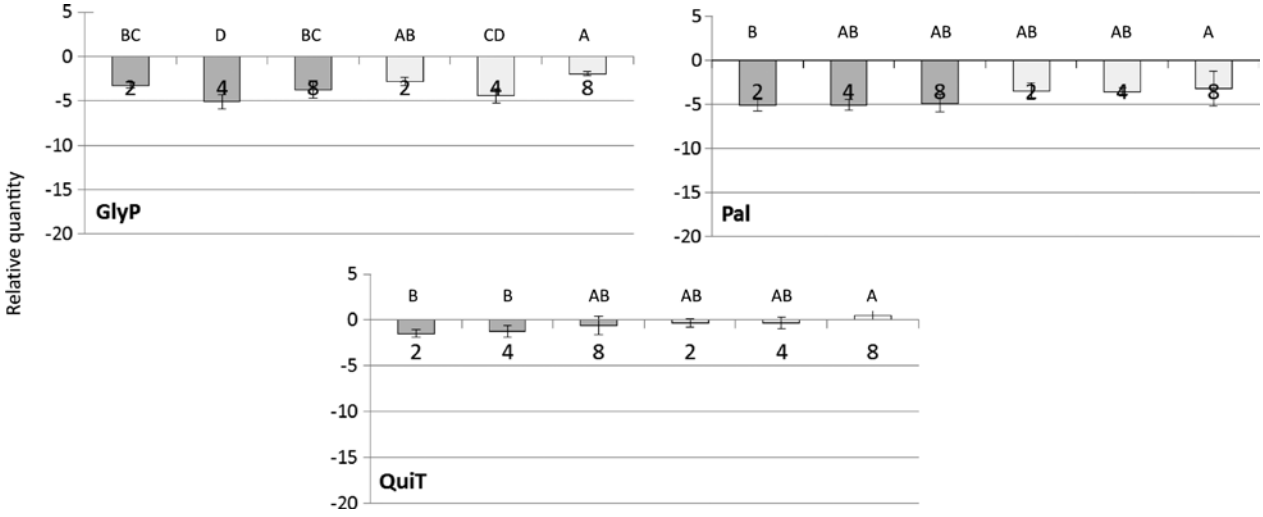

Figure 5: Genes involved in iron reduction and homeostasis.
Mean and StD for tested target genes after 2, 4 and 8 weeks of incubation (n=6). W_{FA} dark grey (left), W light grey (right).

straightforward. Several factors may additionally influence the results such as MC, incubation time, sugar content in the nutrient media, and ML. A time difference in decay severity will occur as W is decayed much faster than W_{FA}. Therefore special attention is given to $W_{FA,8w}$ and W_{2w} because their colonisation levels were well comparable. Several of the target genes in the present study were also explored in studies of *P. placenta* by Martinez et al. (2009) and Vanden Wymelenberg et al. (2010), and for brevity, these two publications will be referred to in the following as Ref. 1 and Ref. 2, respectively. However, a comparison of DNA and RNA retrieval

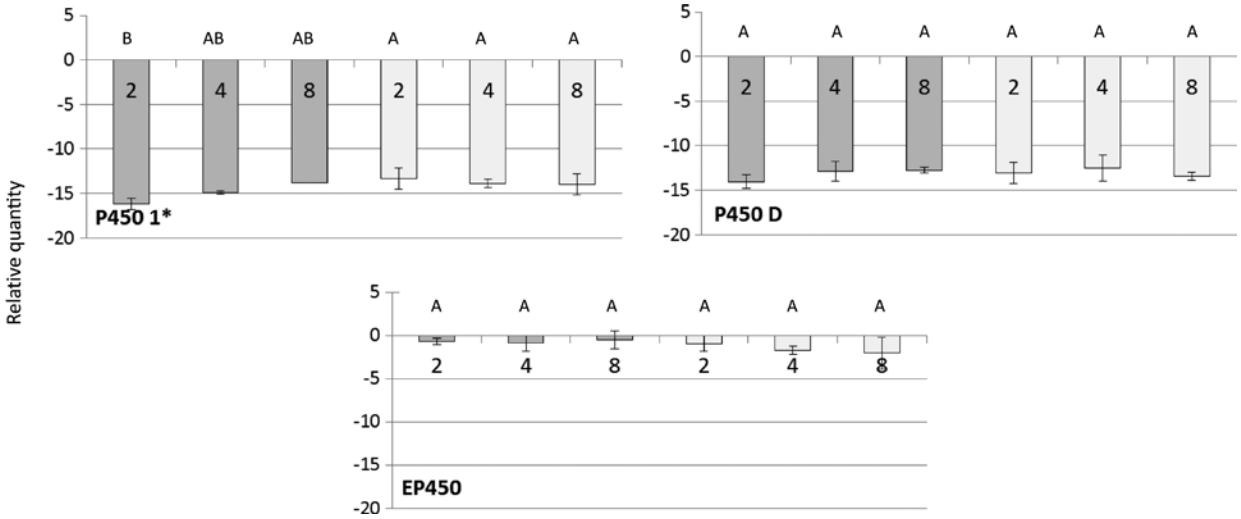

Figure 6: Genes involved in cytochrome P450 monooxygenases.
Mean and StD for tested target genes after 2, 4 and 8 weeks of incubation (n=6). W_{FA} dark grey (left), W light grey (right). The recorded Cq values for *P450* 1 were low, and that only two samples gave measurable values from $W_{FA,4w}$, only one $W_{FA,8w}$. *Significant differences between the variances; O'Brian P=0.04, Brown-Forsythe P=0.04.

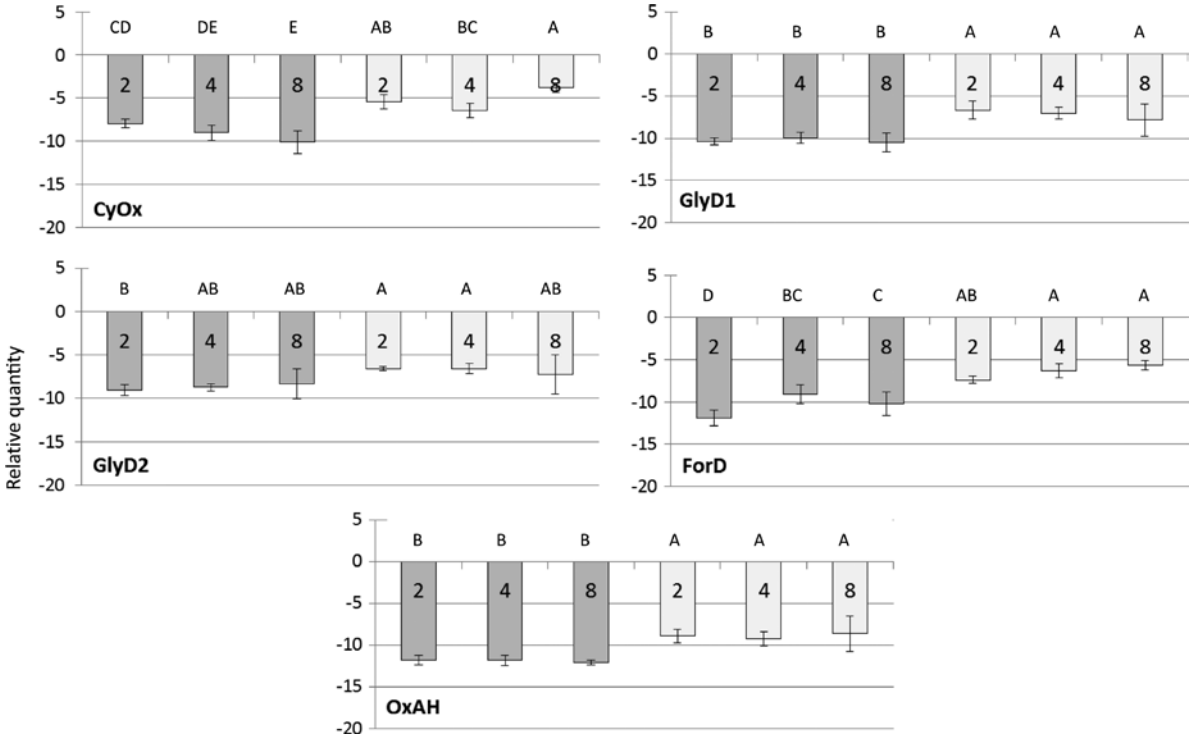

Figure 7: Genes involved with OA.
Mean and StD for tested target genes after 2, 4 and 8 weeks of incubation (n=6). W_{FA} dark grey (left), W light grey (right).

from two different materials is a challenge. From laboratory and field studies (Pilgård et al. 2010) approximately 60% recovery is expected from W_{FA} vs. W. Also the recovery rate of nucleic acid from the wood substrate changes as a function of the degradation level with reduced recovery rates demonstrated at later degradation states. The use of agar plates with malt medium may also affect fungal action within the wood samples. The sugars in the malt are known to inhibit production of some of the enzyme systems, and alternately, they could theoretically promote fungal growth on recalcitrant wood species.

Genes involved in oxidative depolymerisation

During incubation *Lac2* (Pp89382), *MoPhe* (Pp114245), *MPOX1* (Pp44056) and *AlOx1* (Ppl118723) were upregulated slightly in $W_{FA,8w}$ when compared to W_{2w}, the same harvesting point where initial ML was detected. The genes coding for oxidative enzymes in W_{FA} tended to be expressed at a similar, or slightly higher level as compared to W. Expression of these genes never occurred at significantly lower levels in W_{FA} vs. W (Figure 3). Ringman et al. (2014b) found a trend for acetylated, DMDHEU-treated and thermally modified wood showing greater expression of oxidative enzymes in the modified woods vs. untreated wood.

Many brown-rot fungi secrete high levels of OX, which under some conditions will chelate Fe^{3+} tightly. The brown-rot fungi regulate soluble OX production at microsites (Connolly et al. 1996) to promote cell wall depolymerisation when required, and inhibit it when nutrients are not needed (Arantes et al. 2012). The higher pH of the wood cell wall promotes a phase transfer of iron from OX to certain hydroxyquinones (Goodell et al. 1997a; Arantes et al. 2012).

Laccase-catalysed oxidation of lignin model substrates was already demonstrated (Ref. 1). The *P. placenta* results in the quoted work show that Ppl62097 and Ppl111314 are likely laccases sensu stricto (Hoegger et al. 2006). In the current study *Lac1* (Ppl111314) gave similar results in W_{FA} and W and no significant change during incubation (Figure 3). For *Lac2* (Ppl89382) a minor upregulation was found in W_{FA} during incubation, but the variance was not homogeneous and there was no significant difference in expression between $W_{FA,2w}$ and W_{2w} and W_{4w}. Therefore, the overall comparison of *Lac2* is not conclusive.

Here, *AlOx1* (Ppl118723) revealed slightly higher expression levels in W_{FA} than in W. *AlOx1* is similar to *G. trabeum* methanol oxidase (GenBank DQ835989), with >85% amino acid identity over the full sequence length (Ref. 1). Previous immunolocalization studies implicated the *G. trabeum* alcohol oxidase as a potential source of H_2O_2 (Daniel et al. 2007), which would support Fenton chemistry. Microarray analysis in Ref. 1 revealed high transcript levels and a sharp increase in transcription of the gene encoding Ppl118723 in cellulose-grown cultures relative to noncellulolytic cultures, suggesting a similar role in *P. placenta*. In Ref. 2, Ppl118723 exhibited an increased accumulation of transcripts in aspen-grown vs. glucose-grown cultures.

In the current study, *MoPhe* (Ppl114245) was upregulated during incubation in $W_{FA,8w}$, and when comparing $W_{FA,8w}$ with W_{2w}. All other time points and treatments were not significantly different. In Ref. 1, upregulated genes potentially involved in quinone redox-cycling, and oxidation of lignin derived products included *polyphenol oxidase* (Ppl114245), i.e. tyrosinase. In Ref. 2, Ppl114245 was found to be upregulated in aspen amended medium. It has been proposed that phenol oxidase enzymes, in addition to reducing toxic quinones in the fungal environment, are involved in the reduction of quinones in the chelator-mediated Fenton system (Goodell et al. 1997a; Arantes et al. 2012) as the oxidised chelators diffuse back to the cell lumen, and thereby perpetuate Fenton reactions.

Genes involved in enzymatic depolymerisation of polysaccharides

For *βGlu1* (Ppl107557), *βGlu2* (Ppl112501), *GH5* (Ppl117690), and *GH55* (Ppl105490) no significant difference was found between the three harvesting points for neither W_{FA} nor W (Figure 4). For *GH5* and *Gamy* the comparisons are only indicative due to non-homogenous variances. This study indicates that the endoglucanase (*GH5*) and the β-glucosidases (*βGlu1*, *βGlu2*) are down-regulated during growth in W_{FA} compared to W. When comparing $W_{FA,8w}$ with W_{2w} the same three genes are down regulated.

Ref. 2 indicates that *GH5* endoglucanase (Ppl117690) was highly expressed in aspen amended media, but did not exhibit significant transcript accumulation relative to glucose alone. In the same paper, a high constitutive expression was observed of *GH 55* (Ppl105490) for both aspen wood and glucose medium. Family GH15 α-glycosidases are inverting enzymes, as first shown by Weil et al. (1954). Glycoside hydrolases of this family are exo-acting enzymes that hydrolyse the non-reducing end residues of α-glucosides of non-cellulose polysaccharides. Ref. 2 describes that among the glycoside hydrolase-encoding genes only five were upregulated substantially in the medium amended with aspen, the rest of the glycoside hydrolase-encoding genes showed high expression levels both in media amended with glucose and aspen. Tang et al. (2013) found much greater change in *GH5* expression levels than observed in the current study when they compared MCQ preservative treated wood with untreated wood. One explanation can be that gene expression was calculated differently, Tang et al. (2013) calculated expression of the three last harvesting points relative to the first harvesting point (MCQ day 25), and expression values were normalised against 18S rRNA. However, the difference in test design is expected to be the main reason

for the differences, concerning wood protection systems, wood species, test fungus, and incubation times. The magnitude of difference in expression level can be interpreted that copper protection may have a much more active and direct effect on the fungus and thus induce greater transcriptional effects than W_{FA}.

Interestingly, Tang et al. (2013) noticed that when MCQ lost its effectiveness, a number of genes were upregulated, including genes related to degradation of cellulose, hemicelluloses, and pectin. That the carbohydrate degrading machinery is upregulated only when the level of protection was reduced indicate some important similarities with the system in the present study. W_{FA} appears to downregulate some of the *P. placenta* genes involved in enzymatic depolymerisation of polysaccharides during initial decay (W_{FA} still protected), while the oxidative machinery tend to be slightly upregulated. In the current study the aim was to look at the initiation of decay. Hence, a potential change in regulation at more advanced stages of decay of FA protected samples was not monitored, but should be investigated further in another study.

The putatively exo-acting GH15 glucoamylase (*Gamy*) that can depolymerise starch but not cellulose tended to be slightly upregulated in W_{FA} vs. W. The reduced expression of glucoamylase in W possibly reflects the rapid depletion of starch present in the wood after colonisation. It should be noted that the comparison of means for Gamy is hampered with slightly non-homogenous variance (P=0.04). Ringman et al. (2014b) found that for the genes involved in enzymatic degradation (endoglucanase and β-glucosidase), the levels of expression in acetylated, DMDHEU-treated and thermally modified wood were equal to or lower than those in untreated wood.

These results indicate that depolymerisation of polysaccharides by endoglucanase and β-glucosidases may be impaired and may contribute to the lack of decay and ML in W_{FA} samples during the first 8 weeks of incubation in laboratory exposed samples. Hence, supporting Ringman et al. (2014b), there are indications of a possible shift toward slightly increased expression, or at least no down regulation, of genes related to oxidative metabolism and concomitant reduction of the genes related to breakdown of polysaccharides in W_{FA} vs. W. If such a shift exists, it can be interpreted in several ways. It could be an attempt to increase the number of enzymes needed to facilitate breakdown of the modification in order to access the cellulosic source for metabolism. Alternatively, an increase could reflect a stress response brought upon by the modification, lack of access to nutrients or be a combination of all the above mentioned possibilities. However, the growth conditions in this research are atypical relative to those in the natural substrate, and the small differences between gene expression in W and W_{FA} samples complicates the interpretation of the results.

Genes involved in iron reduction and homeostasis

The three genes, *Pal* (Ppl112824), *QuiT* (Ppl44553), and *GlyP* (Ppl128974) showed no significant variation during incubation for W_{FA} and W (Figure 5) except for down regulation of both $W_{FA,4w}$ and W_{4w} for *GlyP*. No significant differences were found between $W_{FA,8w}$ and W_{2w}.

Ref. 1 suggest that the upregulation they found in case of *phenylalanine ammonia lyase* (Ppl112824) and a putative quinate transporter (Ppl 44553) might be because of their roles in biosynthesis and transport of essential quinones, respectively. In the present study, no obvious difference were found in regulation between W_{FA} vs. W for these two genes. In Ref. 2 is stated that "the biosynthesis of hydroquinones, such a 2,5-dimethoxy-1,4-benzoquinone, has not been experimentally established in *P. placenta* but likely involves conversions of aromatic amino acids" and Goodell et al. (1997a) have proposed that they are derived similarly to known catecholate siderophores produced by fungi via secondary metabolism pathways. *Phenylalanine ammonium lyase* (Ppl112824) expression from *P. placenta* was upregulated when the medium was amended with aspen (Ref. 2). In Ref. 1, the gene encoding Ppl128974 was significantly upregulated on microcrystalline cellulose medium.

Genes involved in cytochrome P450 monooxygenases

For *P450-1* (Ppl130132), *P450D* (Ppl128850), and *EP450* (Ppl110015) there were no changes in gene expression during incubation and generally no differences between W_{FA} and W (Figure 6). In Ref. 1, genes encoding Ppl110015 and Ppl128850 were significantly upregulated in cellulose medium. The former is highly conserved in fungi and thought to catalyse benzoate hydroxylation.

Genes linked to oxalic acid (OA)

The general trend was a rather stable gene expression during incubation for both substrates and slightly lower gene expression in W_{FA} than in W (Figure 7). Four of the five genes (*CyOx*, *GlyD1*, *GlyD2*, *ForD* and *OxAH*) had

significantly lower gene expression in $W_{FA,8w}$ than W_{2w} but none of the expression differences observed were large.

It is likely that the increase in OX crystal formation on the surface of hyphae attacking W_{FA} is either related to increased efflux of OX or that post translational aspects, such as activation of the enzymes involved in its production, are more important than their transcription level. Our work did not measure expression of additional potentially relevant enzymes involved in OX synthesis, destruction or the interface of the TCA and glyoxylate cycles. Other explanations could be that a lack of moisture and/or relatively high metal ion concentration in the wood limits the diffusion of OX away from the hyphae and/or variation in the ratio of soluble OX vs. insoluble OX over time.

pH

According to Kebony (Stig Lande personal communication), the pH value of W_{FA} powder in water is in the range of 3.9–4.3 depending on the specific product and sampling method. In the current study, the pH for W_{FA} was 3.11±0.01 for non-decayed samples and 3.11±0.06 for $W_{FA,8w}$. Hence, the pH was stable during incubation. Among characteristics of brown-rot are release of OA and lowering of the local pH, often to about pH 2 (Hyde and Wood 1997). The pH of the microenvironment immediately around the fungus is low, but the wood cell wall is highly buffered and typically remains at about pH 5.5. As Fe-OX complexes move into the wood cell wall, iron can be sequestered from OX in a phase transfer type reaction. Once iron is chelated by hydroxyquinone chelators produced by the fungus, but within the wood cell wall, the iron is immediately reduced and hydroxyl radicals can be generated in a chelator-mediated Fenton reaction (Goodell et al. 1997a; Arantes et al. 2012). This mechanism permits generation of hydroxyl radicals within the wood cell wall, limiting generation of these radicals near the fungal hyphae as it would be destructive to the fungus. It is possible that FA treatment interferes in this manner so that a micro pH differential develops, and this may be one reason why the FA treatment is effective. However, more detailed micro pH determinations are needed in this regard.

Conclusions

Study of early *P. placenta* colonisation and decay in W_{FA} and W during 8 weeks of incubation revealed that:

- Elongated clusters of long crystals, square in cross-section, were formed abundantly on the *P. placenta* hypha during colonisation of W_{FA}. EDX and morphological appearance indicate that these are consistent Ca-OX crystals. This type of crystal was not observed during colonisation of W.
- There were indications of a possible shift toward increased, or at least no down regulation of the gene expression related to oxidative metabolism and concomitant reduction of expression of some of the genes related to breakdown of polysaccharides in W_{FA} compared to W. Also for some of the genes involved with OA processes there was a slight down regulation in W_{FA} compared to W.
- Selected cytochrome P450 monooxygenases and genes involved in iron reduction and homeostasis did not vary much during incubation or between W_{FA} and W.

Acknowledgments: Sigrun Kolstad and Inger Heldal are acknowledged for the molecular analyses and Monica Fongen for TGA, pH and oxalate analysis. This project was financed by The Research Council of Norway (179482/I30 Wood degradation, 243663/E50 BioMim) and SGB No. 335008. Goodell was also supported in part by the USDA-HATCH Project S-1041 VA-136288.

References

AWPA (2001) Standard method of testing wood preservatives by laboratory soil-block cultures. American Wood Protection Association (E-10).

Arantes, V., Goodell, B. (2014) Current understanding of brown-rot fungal biodegradation mechanisms: A Review. In: Deterioration and Protection of Sustainable Biomaterials. Eds. Nicholas, D.D., Goodell, B., Schultz, T. ACS Series. Oxford University Press, New York, USA, Vol. 1158, pp. 3–21.

Arantes, V., Milagres, A.M., Filly, T.R., Goodell, B. (2011) Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: the relevance of nonenzymatic Fenton-based reactions. J. Ind. Microbiol. Biot. 38:541–555.

Arantes, V., Jellison, J., Goodell, B. (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl. Microbiol. Biotechnol. 94:323–338.

Baldrian, P., Valaskova, V. (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol. Rev. 32:501–521.

Bosshard, H.H., Hug, U.E. (1980) The anastomoses of the resin canal system in *Picea abies* (L.) Karst, *Larix decidua* Mill and *Pinus sylvestris* L. Holz Roh Werkst. 38:325–328.

Boonstra, M.J., Tjeerdsma, B. (2006) Chemical analysis of heat treated softwoods. Holz. Roh. Werkst. 64:204–211.

Boonstra, M.J., Van Acker, J., Kegel, E., Stevens, M. (2007) Optimisation of a two-stage heat treatment process: durability aspects. Wood Sci. Technol. 41:31–57.

Bravery, A.F. A miniaturised wood-block test for the rapid evaluation of preservative fungicides. Proceedings of a special seminar held in association with the 10th annual meeting of the IRG,

Peebles, Rep. No. 136. Swedish Wood Preservation Institute, Stockholm, 1979.
Chuang, I.S., Maciel, G.E., Myers, G.E. (1984) Carbon-13 NMR study of curing in furfuryl alcohol resins. Macromolecules 17:1087–1090.
Connolly, J.H., Jellison, J. (1995) Calcium translocation, calcium oxalate accumulation, and hyphal sheath morphology in the white-rot fungus *Resinicium bicolor*. Can. J. Bot. 73:927–936.
Connolly, J.H., Arnott, H.J., Jellison, J. (1996) Patterns of calcium oxalate crystal production by three species of wood decay fungi. Scanning Microsc. 10:385–400.
Daniel, G., Volc, J., Filonova, L., Plihal, O., Kubatova, H.P. (2007) Characteristics of *Gloeophyllum trabeum* alcohol oxidase, an extracellular source of H_2O_2 in brown rot decay of wood. Appl. Environ. Microbiol. 73:6241–6253.
Eastwood, D.C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., Asiegbu, F.O., Baker, S.E., Barry, K., Bendiksby, M., Blumentritt, M., Coutinho, P.M., Cullen, D., de Vries, R.P., Gathman, L., Goodell, B., Henrissat, B., Ihrmark, K., Kauserud, H., Kohler, A., LaButti, K., Lapidus, A., Lavin, J.L., Lee, Y.-H., Lindquist, E., Lilly, W., Lucas, S., Morin, E., Murat, C., Oguiza, J.A., Park, J., Pisabarro, A.G., Riley, R., Rosling, A., Salamov, A., Schmidt, O., Schmutz, J., Skrede, I., Stenlid, J., Wiebenga, A., Xie, X., Kües, U., Hibbett, D.S., Hoffmeister, D., Högberg, N., Martin, F., Grigoriev, I.V., Watkinson, S.C. (2011) The plant cell wall–decomposing machinery underlies the functional diversity of forest fungi. Science 333:762–765.
Eriksson, K.-E.L., Blanchette, R.A., Ander, P. Microbial and Enzymatic Degradation of Wood and Wood Components. Springer-Verlag Berlin Heidelberg, Germany, 1990.
CEN (1996a) EN 113. Wood preservatives – Test method for determining the protective effectiveness against wood destroying basidiomycetes – Determination of toxic values. Brussels, Belgium, European Committee for Standardization.
CEN EN 84. Wood preservatives. Accelerated ageing of treated wood prior to biological testing – Leaching procedure. Brussels: European Committee for Standardisation.
Esteves, B., Pereira, H.M. (2009) Wood modification by heat treatment: a review. Bioresources 4:370–404.
Goodell, B. (2003) Brown rot degradation of wood: Our evolving view. In: Wood Deterioration and Preservation: Advances in Our Changing World. Eds: Goodell, B., Nicholas, D., Schultz, T. American Chemical Society Series. Oxford University Press, New York, USA, pp. 97–118.
Goodell, B., Jellison, J., Liu, G., Daniel, A., Paszczynski, F., Fekete, Krishnamurthy, S., Jun, L., Xu, G. (1997a) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. J. Biotechnol. 53:133–162.
Goodell, B., Daniel, G., Liu, J., Mott, L., Frank, R. (1997b) Decay resistance and microscopic analysis of wood-cement composites. Forest Prod. J. 47:75–80.
Goodell, B., Jellison, J. Loferski, S.L. Quarles (2007) Brown-rot decay of ACQ and CA-B treated lumber. Forest Prod. J. 57:31–33.
González, R., Martinez, R., Ortiz, P. (1992) Polymerization of furfuryl alcohol with trifluoroacetic-acid – the influence of experimental conditions. Macromol. Chem. 193:1–9.
González, R., Figueroa, J.M., González, H. (2002) Furfuryl alcohol polymerization by iodine in methylene chloride. Eur. Polym. J. 38:287–297.
Hietala A.M., Stefańczyk, E., Nagy, N.E., Fossdal C.G., Alfredsen G. (2014). Influence of wood durability on the suppressive effect of increased temperature on wood decay by the brown-rot fungus *Postia placenta*. Holzforschung 68:123–131.
Hill, C.A.S. Wood modification: chemical, thermal and other processes. John Wiley & Sons, Ltd., Sussex, UK, 2006.
Hill, C.A.S. (2009) Why does acetylation protect wood from microbiological attack? Wood Mater. Sci. Eng. 4:37–45.
Hill, C.A.S. (2011) Wood modification: an update. BioResources 6: 918–919.
Hill, C.A.S., Forster, S.C., Farahani, M.R.M., Hale, M.D.C., Ormondroyd, G.A., Williams, G.R. (2005) An investigation of cell wall micropore blocking as a possible mechanism for the decay resistance of anhydride modified wood. Int. Biodeter. Biodegr. 55:69–76.
Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R., Kües, U. (2006) Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J. 273:2308–2326.
Hyde, S.M., Wood, P.M. (1997) A mechanism for production of hydroxyl radicals by the brown-rot fungus *Coniophora puteana*: Fe(III) reduction by cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae. Microbiology 143:259–266.
Jarosz-Wilkołazka, A., Gadd, G.M. (2003). Oxalate production by wood-rotting fungi growing in toxic metal-amended medium. Chemosphere 52:541–547.
Kang, Y.M., Prewitt, L., Diehl, S. (2009) Gene expression of selected decay enzymes produced during biodeterioration of three wood types. International Research Group on Wood Preservation, 40th Annual Conference, Beijing, China, IRG/WP 09-10702.
Lande, S. (2008) Furfurylation of wood – Wood modification by the use of furfuryl alcohol. PhD thesis. Norwegian University of Life Science. Ås, Norway. ISBN 978-82-575-0814-2. ISSN 0802-3220.
Lande, S., Westin, M., Schneider, M. (2004) Eco-efficient wood protection-furfurylated wood as alternative to traditional wood preservation. Management of Environmental Quality: An Int. J. 15:529–540.
Lande, S., Eikenes, M., Westin, M., Schneider, M. (2008) Furfurylation of wood: chemistry; properties and commercialization. In: Development of Commercial Wood Preservatives. Eds. Schultz, T.P., Militz, H., Freemann, M.H., Goodell, B., Nicholas D.D. ACS Symposium Series 982, pp 337–355.
Maciel, G.E., Chuang, I.S., Myers, G.E. (1982) Carbon-13 NMR study of cured furfuryl alcohol resins using cross polarization and magic-angle spinning. Macromolecules 15:1218–1220.
Martinez, D., Challacombea, J., Morgensternc, I., Hibbettc, D., Schmolld, M., Kubicekd, C.P., Ferreirae, P., Ruiz-Duenase, F.J., Martineze, A.T., Kerstenf, P., Hammelf, K.E., Vanden Wymelenberg, A., Gaskellf, J., Lindquisth, E., Sabati, G., BonDuranti, S.S., Larrondoj, L. F., Canessaj, P., Vicunaj, R., Yadavk, J., Doddapanenik, H., Subramaniank, V., Pisabarrol, A. G., Lavínl, J.L., Oguizal, J.A., Masterm, E., Henrissatn, B., Coutinhon, P.M., Harriso, P., Magnusonp, J.K., Bakerp, S.E., Brunop, K., Kenealyq, W., Hoegger, P.J., Kuesr, U., Ramaiyao, P., Lucash, S., Salamovh, A., Shapiroh, H., Tuh, H., Cheeb, C.L., Misraa, M., Xiea, G., Tetero, S., Yavero, D., Jamess, T., Mokrejst, M., Pospisekt, M., Grigorievh, I.V., Brettina, T., Rokhsarh, D., Berkao, R., Cullenf, D. (2009) Genome, transcriptome, and secretome analysis of wood decay

fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc. Natl. Acad. Sci. 106:1954–1959.

Niemenmaa, O., Uusi-Rauva, A., Hatakka, A. (2007) Demethoxylation of [O(14)CH (3)]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria* (*Postia*) *placenta*. Biodegradation 19:555–565.

Papadopoulos, A.N. (2010) Chemical modification of solid wood and wood raw material for composites production with linear chain carboxylic acid anhydrides: a brief review. BioResources 5:499–506.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:2002–2007.

Pilgård, A., Alfredsen, G., Hietala, A. (2010) Quantification of fungal colonization in modified wood – qPCR as a tool for studies on *Trametes versicolor*. Holzforschung 64:645–651.

Pilgård, A., Alfredsen, G., Björdal, C.G., Børja, I., Fossdal, C.G. (2011) qPCR as a tool to study basidiomycete colonization in wooden field stakes. Holzforschung 65:889–895.

Punja, Z.K., Huang, J.-S., Jenkins, S.F. (1985) Relationship of mycelial growth and production of oxalic acid and cell wall degrading enzymes to virulence in *Sclerotium rolfsii*. Can. J. Plant Pathol. 7:109–117.

Rahman, M.M., Niimi, M., Kawamura, O. (2007) Simple method for determination of oxalic acid in forages using high-performance liquid chromatography. JPN Soc. Grassland Sci. 53:201–204.

Ringman, R., Pilgård, A., Brischke, C., Richter, K. (2014a) Mode of action of brown rot decay resistance in modified wood: a review. Holzforschung 68:239–246.

Ringman, R., Pilgård, A. Richter, K. (2014b) Effect of wood modification on gene expression during incipient *Postia placenta* decay. Int. Biodeter. Biodegr. 86:86–91.

Rowell, R.M. Wood Chemistry and Wood Composites. Taylor & Francis 2005.

Rowell, R.M., Ibach, R.E., McSweeny, J., Nilsson, T. (2009a) Understanding decay resistance, dimensional stability and strength changes in heat-treated and acetylated wood. Wood Mater. Sci. Eng. 4:14–22.

Rowell, R.M., Ibach, R.E., McSweeny, J. Nilsson, T. (2009b) Understanding decay resistance, dimensional stability and strength changes in heat treated and acetylated wood. In: Proceedings of The Fourth European Conference on Wood Modification (ECWM4), Stockholm, Sweden, pp. 489–502.

Rozen, S., Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365–86.

Sayer, J.A., Raggett, S.L., Gadd, G.M. (1995) Solubilization of insoluble metal compounds by soil fungi: development of a screening method for solubilizing ability and metal tolerance. Mycol. Res. 99:987–993.

Schilling, J.S., Jellison, J. (2004) High-performance liquid chromatographic analysis of soluble and total oxalate in Ca- and Mg-amended liquid cultures of three wood decay fungi. Holzforschung 58:682–687.

Tang, J.D., Parker, L.A., Perkins, A.D., Sonstegard, T.S., Schroeder, S.G., Nicholas, D.D., Diehl, S.V. (2013) Gene expression analysis of copper tolerance and wood decay in brown rot fungus *Fibroporia radiculosa*. App. Environ. Microbiol. 79:1523–1533.

van Riel, S., Lande, S., Larnøy, E., Eikenes, M. (2007) Quality control of furfurylated Scots pine by TGA. Proceedings of The Third European Conference on Wood Modification (ECWM3), Cardiff, UK, pp. 423–430.

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Martinez, D., Grigoriev, I., Kersten, P.J., Cullen, D. (2010) Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 76:3599–3610.

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., BonDurant. S.S., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Grigoriev, I.V., Kersten, P.J., Cullen, D. (2011) Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 77:4499–4507.

Venås, T.M. (2008) A study of mechanisms related to the fungal decay protection rendered by wood furfurylation. PhD thesis, University of Copenhagen.

Verma, P., Mai, C. (2010) Hydrolysis of cellulose and wood powder treated with DMDHEU by a hydrolase enzyme complex, Fenton's reagent, and in a liquid culture of *Trametes versicolor*. Holzforschung 64:69–75.

Weil, C.E., Burch, R.J., Van Dyk, J.W. (1954) An α-amyloglucosidase that produces β-glucose. Cereal. Chem. 31:150–158.

Xu, G., Goodell, B. (2001) Mechanisms of wood degradation by brown-rot fungi: Chelator-mediated cellulose degradation and binding of iron by cellulose. J. Biotechnol. 87:43–57.

Yelle, D.J., Ralph, J., Lu, F., Hammel, K.E. (2008) Evidence for cleavage of lignin by a brown rot basidiomycete. Environ. Microbiol. 10:1844–1849.