

Fungal colonisation of outside weathered modified wood

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Abstract Specimens of Scots pine sapwood (*Pinus sylvestris* L.) and beech wood (*Fagus sylvatica* L.) were treated with an amino-alkyl-functional oligomeric siloxane, a sodium water glass solution and 1,3-dimethylol-4,5-dihydroxyethylene urea (DMDHEU). Treated and untreated wood specimens were exposed outdoors without ground contact. After 9 months of outside exposure, all specimens showed discolouration caused by infestations of mould and staining fungi on the exposed wood surface. Fungi grown on the sample surface were isolated and identified by microscopic technique and sequencing of PCR-amplified DNA from the ITS region. Primarily, an infestation by ascomycetes and related deuteromycetes was found. The most dominant fungi were *Trichoderma* sp. and *Epicoccum* sp.. An infestation of *Aureobasidium pullulans* was only detected on untreated and DMDHEU-treated samples. There were only marginal differences of fungal infestation between the two wood species.

Introduction

Wood is used as material for many outside applications with and without ground contact. Examples for applications without ground contact under use class 3 conditions according to EN 335-1 (2006) are claddings, decking, noise barriers or garden furniture. Decay fungi, moulds and blue stain fungi are able to colonise wood in those applications. Fungal spores are omnipresent in the environment, but the occurrence and amount of spores rely on environmental influences.

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The process of colonisation depends on the environmental conditions and on the microclimate on the sample surface, which changes permanently during outside exposure. The fungal infestation of wood is a complex process including interactions between fungi, wood and environmental conditions (Deacon 1997). The germination of spores depends on the wood moisture content, temperature, accessible wooden substrate and nutrients (Eaton and Hale 1993; Schmidt 2006). Early colonizers of wood are mainly blue stain and mould fungi (Clubbe 1980; Dix and Webster 1995).

They cause discolouration, but no significant mass loss and generally do not affect the structural properties of wood (Liese and Schmid 1961; Grosser 1985; Zink and Fengel 1988). Free water in the lumens of wood cells over longer periods is needed for the growth of sap-staining fungi.

In the last decades, very effective biocidal treatments were used to protect wood in outside applications. A biocide treatment has a direct impact on the fungal organisms, such as denaturation of proteins, inhibition of protein synthesis or cell membrane disruption (Eaton and Hale 1993). Beside biocide treatment, new wood modification strategies for wood protection have been developed. In comparison with biocidal treatments, these new strategies focus on a change in the material properties of wood to prevent the colonisation by fungi, such as reduction of moisture content and changes in the chemical structure of wood (Hill 2006). Most of the research in chemical modification of wood was done to improve the biological resistance against wood-decaying organisms, while staining reduction was not in the main focus. Fungal colonisation of modified wood particularly in early stages might differ from that of traditionally biocide-treated one, because of a different mode of action against wood-inhabiting organisms. In various studies, identification of fungi from modified and preservative-treated wood was described. In studies by Raberg et al. (2006) on wood panels exposed outdoors at a test field in Uppsala (Sweden), thermally treated Norway spruce specimens were mainly infested by *Mucor* sp. and *Hormonema dematioides* and wood panels of acetylated Scots pine sapwood specimens by *Cladosporium* sp. and *Phoma leveillei*. Investigations by Choi et al. (2003) in Vancouver (Canada) on copper chromium arsenate (CCA)-treated deckings outside exposed for 0.4–20 years reported an infestation of bacteria, mould and staining fungi, soft rot and basidiomycetes. In this investigation, the most dominant genus was *Phialophora* sp. Moreover, a relatively low infestation of *Trichoderma* sp. was observed in this study. Soft rot fungi and basidiomycetes were isolated mostly on the long-term exposed specimens.

Different techniques exist to detect fungal species growing in wood. Morphological identification according to taxonomic guides by cultural characteristics is the traditional way (von Pechmann et al. 1964; Nobles 1965; Wilcox and Dietz 1997). Several molecular methods have been developed in the last years to identify fungi on wood, for example sequence analyses of rDNA regions and fungus-specific amplification of known internal transcribed spacer (ITS) sequences by the polymerase chain reaction (Moreth and Schmidt 2000; Schmidt and Moreth 2002; Hoegger and Kües 2007).

In this study, 1,3-dimethylol-4,5-dihydroxyethylene urea (DMDHEU), an amino-alkyl-functional oligomeric siloxane and a sodium water glass solution were used

for the chemical modification of pine and beech wood. Previous work indicated that treatments with DMDHEU and siloxanes can restrict but not prevent an infestation of blue stain during outside weathering (Xie 2005; Donath 2004). Water glass treatments resulted after 3 years in above ground weathering tests in lack of infestation by blue stain fungi (Dellith 2006). Little knowledge exists on the type of fungal species that are able to colonise the wood surfaces treated with these chemicals. The objective of this study was therefore to determine the fungal species on the surfaces of modified wood kept for 9 months in outside weathering conditions without ground contact. Morphological characteristics and sequencing of PCR-amplified DNA from the ITS regions of rRNA gene clusters were used for fungal identification.

Materials and methods

Treatment of the wood specimens

Specimens of Scots pine sapwood (*Pinus sylvestris* L.) and beech (*Fagus sylvatica* L.) free of knots and cracks were prepared with a size of $150 \times 74 \times 18 \text{ mm}^3$ (longitudinal \times tangential \times radial). The chemicals used in this study for wood modification are described in Table 1.

Impregnation of wood specimens was carried out by applying a vacuum of 60 mbar (30 min) and a subsequent pressure of 12 bar (2 h). All treatments were carried out in a laboratory-scale process. After impregnation, siloxane-impregnated specimens were pre-dried at 40°C (4day). Curing of the siloxane was subsequently performed at 103°C (24 h).

The water glass-treated specimens were stored for 3 weeks in a desiccator under carbon dioxide atmosphere, which was established by floating the desiccator in regular steps with CO₂ from a gas bomb. DMDHEU-impregnated specimens were cured in a hot steam dryer.

Outside exposure

Prior to outside exposure, specimens of Scots pine sapwood and beech wood were conditioned in a climate chamber at 20°C/65% relative humidity (RH). Subsequently, the specimens were placed and fixed on weathering racks with a 45° slope

Table 1 Chemicals

Chemical characterisation	Trade name	Concentration
Sodium water glass with additives	BETOL 39 T3 (Woellner, Ludwigshafen, Germany)	15 wt/wt
Amino-alkyl-functional oligomeric siloxane	DYNASYLAN [®] HS 2909 (Evonik, Rheinfelden, Germany)	20 wt/wt
N-methylol compound, 1,3-dimethylol-4,5-dihydroxyethyleneurea (catalyst MgCl ₂)	DMDHEU (BASF, Ludwigshafen, Germany)	1.3 M

direction towards south-west. The weathering racks were located at a field on the grounds of the University of Goettingen. Ten specimens per treatment and ten untreated specimens were exposed and after 3 months first time inspected for fungal infestations. The surface of each five of them was analysed for fungal infestation on the surface after 9 months (July to March) of outside exposure.

Identification of fungi

Fungi were isolated from the weathered side of the exposed specimens. Parts of the wood specimens that showed visible signs of staining were removed from the surface with sterile tools and placed onto 2% malt extract agar (MEA, Scharlau Microbiology, Barcelona, Spain) plates. Plates were incubated at 22°C and 70% RH for several weeks. Fungi growing on MEA were transferred onto fresh MEA plates to obtain pure cultures by further subculturing.

Microscopic analysis

All plates were observed for macroscopic characteristics such as colour of mycelia, colour of mycelium, colour of the reverse side of the mycelium and growth rate of fungal culture. Microscopic characteristics, such as hyphal features and spore properties (size, shape, colour), were observed on slides using an Eclipse E 600 light microscope (Nikon Germany, Düsseldorf, Germany). For fungal identification, taxonomic guides were used (Funder 1953; Arx von 1981; Larone 1995; Watanabe 2002; Dugan 2006). The microscopic analysis was performed with stained (lactophenol blue) and unstained species of the fungal culture.

PCR analyses

ITS sequence characterisation was used for fungal identification. The DNA was extracted from fungal mycelium as described in rapid DNA mini preparation protocol by Liu et al. (2000). PCR was performed in a total volume of 25 µl containing 10 mM Tris pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany), each 0.4 µM of primer and 1 U of Taq DNA polymerase. The standard primers for ITS amplification in fungi (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) were used. PCR conditions were 2-min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C and a final extension at 72°C for 10 min. For sequencing, PCR products were precipitated with 35 µl isopropanol, dried and resuspended in 20 µl H₂O. For cycle sequencing reactions, 2 µl of the DNA were mixed with BigDye Terminator v3.1 (Applied Biosystems, Darmstadt, Germany) applying one-fourth of the reagents recommended by the manufacturer. The same primers as for PCR were separately used in sequencing reactions. After cycling following the manufacturer's instructions, the reaction products were purified by sodium acetate/ethanol precipitation and washed with 70% ethanol. The reaction was run on an ABI PRISM 3,100 Genetic Analyzer (Applied Biosystems). The obtained sequences

from the different fungal mycelia were assembled by using the STADEN PACKAGE program and searched in the NCBI GenBank using the BLAST search function. Obtained sequences were recorded at NCBI under accession numbers GU433218 to GU 433226.

Results and discussion

Outside exposure

All chemical treatments restricted the fungal infestation at the beginning of outside exposure (3 months) compared to untreated specimens. However, all specimens showed visible surface discolouration after 9-month outside exposure caused by a fungal infestation of mould and stain on the exposed wood surface. The untreated specimens of Scots pine sapwood displayed the highest surface discolouration and the water glass-treated samples of Scots pine sapwood the lowest discolouration. Signs of fungal decay were not visible on treated and untreated wood specimens.

A radial penetration of fungal hyphae was reduced, but fungal growth of the exposed surface of the specimens was clearly visible. However, the fungal penetration into the wood tissue is different between treated and untreated wood. DMDHEU treatment of wood reduced the penetration depth of fungi such as *Aureobasidium pullulans* into wood and siloxane as well as water glass treatment fully blocked the invasion (Xie et al. 2008; Pfeffer et al. 2010). Moisture gradients exist in wood from the outer to the inner zones (Eaton and Hale 1993). In the DMDHEU- and siloxane-treated samples, the moisture content of the exposed surfaces might be lower because of the presence of chemicals on the surface as well as in the main penetration paths for liquid water, which block the transfer of water into the inner zones of the wood (Donath et al. 2006, 2007; Xie et al. 2008) and, consequently, restricts the growth of fungi.

Identification of fungi

After 9 months of outside exposure, fungal mycelia were isolated from all specimens of Scots pine and beech wood by using agar medium. Pure cultures of morphological distinct isolates were incubated for 6–8 weeks required for some of the isolates for efficient sporulation. Five of the analysed fungi could not be classified by lack of spores. Hyphae and mitospore characteristics were used to identify the other isolates by microscopic characteristics, such as exemplary shown in Fig. 1 for conidia of *Lewia* sp. and Fig. 2 for macroconidia of *Fusarium* sp.

The sporulating fungi were found to belong to seven different genera of the ascomycetes and related deuteromycetes (fungi imperfecti). Only one isolate (*A. pullulans*) was identified at the species level (see Tables 2 and 3).

There were only marginal differences of fungal infestation between the two wood species. The most dominant fungi in this study were *Trichoderma* sp. and *Epicoccum* sp.. *Trichoderma* sp. was isolated from all test specimens independent of treatment and wood species. An infestation of *A. pullulans* was only detected on



Fig. 1 Conidia of *Lewia* sp., 400×

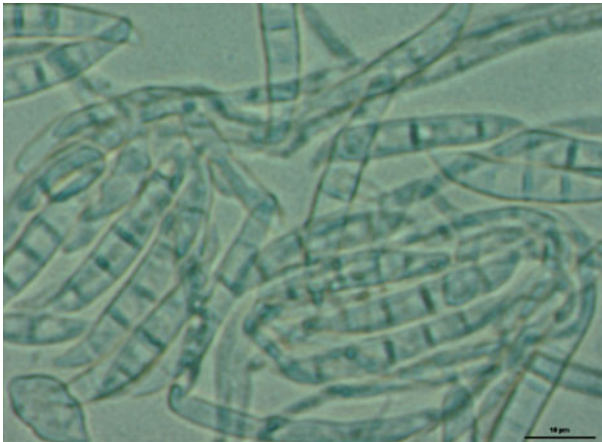


Fig. 2 Macroconidia of *Fusarium* sp., 1,000×

untreated and DMDHEU-treated specimens. Other fungi occurred irregularly on wood specimens without a recognisable preference for wood type or treatment of wood (see Table 4).

In the following, fungal isolates taken from Scots pine specimens were selected for molecular identification. In all instances, ITS sequences obtained from the fungi enabled the identification of a fungal genus. The molecular identification confirmed the genera identification of the microscopic observations.

BLAST searches in the NCBI database showed that the sequences of the isolates had a homology of minimal 90% with the best matching sequences. By sequence hits, five fungi including *A. pullulans* could be identified at the species level. Furthermore, the formerly not definable isolates were determined by ITS sequences as *Peniophora* sp., *Hypocrea* sp. and *Diplodia* sp., respectively.

Table 2 Fungi isolated from Scots pine sapwood specimens and identified by microscopic analysis

No.	Fungal identity	Treatment			
		Untreated	Siloxane	Water glass	DMDHEU
1	<i>Trichoderma</i> sp.	x	x	x	x
2	<i>Epicoccum</i> sp.	x	x	x	x
3	<i>Aureobasidium pullulans</i>	x			x
4	<i>Lewia</i> sp.			x	
5	<i>Chaetomium</i> sp.				x
6	<i>Fusarium</i> sp.		x	x	
7	Unknown	x			
8	Unknown			x	
9	Unknown		x		

x Fungus isolated

Table 3 Fungi isolated from beech specimens and identified by microscopic analysis

No.	Fungal identity	Treatment			
		Untreated	Siloxane	Water glass	DMDHEU
1	<i>Trichoderma</i> sp.	x	x	x	x
2	<i>Epicoccum</i> sp.		x	x	
3	<i>Aureobasidium pullulans</i>	x			x
4	<i>Lewia</i> sp.			x	
5	<i>Chaetomium</i> sp.	x			
6	<i>Penicillium</i> sp.	x			
7	Unknown			x	
8	Unknown		x		

x Fungus isolated

Table 4 Fungi isolated from Scots pine and identified by ITS sequence analysis

No.	GenBank accession	GenBank best hit	Fungal identity	Sequence identity (%)	Query coverage of sequence (%)
1	GU433218	AJ 279448	<i>Epicoccum nigrum</i>	99	100
2	GU433219	AY 853249	<i>Aureobasidium pullulans</i>	97	97
3	GU433220	AF 456917	<i>Trichoderma atroviride</i>	100	99
4	GU433221	AF 397240	<i>Lewia infectoria</i>	100	95
5	GU433222	EF 524036	<i>Chaetomium globosum</i>	100	99
6	GU433223	FJ 614630	<i>Fusarium</i> sp.	100	100
7	GU433224	AY 208796	<i>Peniophora</i> sp.	97	98
8	GU433225	FJ 442679	<i>Hypocrea</i> sp.	95	100
9	GU433226	EU 650671	<i>Diplodia</i> sp.	98	90

In summary, a collection of 10 different species of ascomycetes and related deuteromycetes was isolated from chemically modified Scots pine sapwood and beech wood specimens as well as untreated samples after 9-month outside exposure. All fungi detected in this study are ubiquitous and have previously been reported as mould and stain fungi on exposed surfaces of treated and untreated wood species at different climatic conditions (Choi et al. 2003; Raberg et al. 2006, 2007; Cabrera et al. 2009). Clubbe (1980) stated that bacteria, primary moulds and staining fungi are the initial colonists in untreated Scots pine, substituted in time first by soft rots and then basidiomycetes and cellulolytic secondary moulds. Other authors differentiated infestation by primary moulds in the beginning, followed by infestation of staining and soft rot fungi and then secondary moulds and in the latest stage infestation by wood-degrading basidiomycetes (Käärik 1983; Dix and Webster 1995; Deacon 2006). According to these schemes, the fungal infestation of this study corresponds to the initial stage where mould and stain fungi are the main colonizers.

The composition of fungal species on outside-exposed wood specimens is influenced by the material itself and the type of treatment it had, by climatic influences (surrounding temperature, relative humidity, rainfall) and exposure time and the influence of the general local environment on the microbial biodiversity (Käärik 1983; Eaton and Hale 1993).

On wood, interactions between fungal species have also a strong impact on the composition of fungal infestation (Dix and Webster 1995). The most dominant fungus *Trichoderma atroviride* in this study is a mycoparasitic species that may grow necrotrophically on diverse fungi on wood and bark and act in biocontrol of sapstain species (Schubert et al. 2008). *Epicoccum nigrum*, the second most often found species in this study, has also antagonistic properties against the growth of other fungi (Madrigal et al. 1994), and related species have been shown to delay wood decay by xylophagus basidiomycetes (Mielnichuk and Lopez 2007). It is therefore possible that these two fungi restricted the growth of other species during outside exposure of the wood specimens of this study.

However, well-known technical problems during isolation may result in a biased spectrum of isolates which cannot be fully excluded in this study. For instance, slow-growing mycelium can be easily overgrown during the isolation steps by more rapidly growing mycelia. *Trichoderma atroviride* on the one hand is capable of very fast growth (Jaklitsch 2009) whereas on the other hand, *Aureobasidium pullulans* is only growing at a slower rate (Butin 1964). Likewise, fast-growing non-decay fungi can overgrow slower wood basidiomycete decay fungi (Dix and Webster 1995). Basidiomycete decay fungi were not isolated in this study, not even from untreated wood specimens, either due to such technical deficiencies or because such late fungi in wood infestation were not yet present in the wood.

Further investigations after longer periods of outside exposure will be necessary to get more detailed information about the changes of fungal species and schemes of fungal infestations on siloxane-, water glass- and DMDHEU-treated wood in comparison with untreated wood.

Conclusion

Wood specimens treated with a sodium water glass solution, a siloxane and DMDHEU showed after 9-month outside exposure a surface discolouration caused by mould and staining fungi while there were no signs of decay. All fungi detected in this study on wood surfaces are ubiquitous and have previously been reported on exposed wood surfaces. There were only marginal differences of species distribution between the two wood species. But the fungal distribution and penetration into the wood were different between treated and untreated wood. *A. pullulans* that typically causes blue stain in service on outside-exposed wooden surfaces was only detected on untreated and DMDHEU-treated specimens, but the fungal penetration into the wood tissue was strongly reduced in DMDHEU-treated specimens. Basidiomycetes decay fungi were not isolated in this study.

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