MIGRATION OF BLUE STAIN FUNGI WITHIN WAX IMPREGNATED WOOD

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SUMMARY

The colonization of wax impregnated pine sapwood (Pinus sylvestris L.) and beech (Fagus sylvatica L.) by blue stain fungi and their hyphal migration within the treated wood was investigated. Neither the hydrophobation effect nor the physical presence of the congealed wax deposits could impede fungal growth on the surface. However, in contrast to the untreated controls, there was a lower occurrence of hyphae in wax-treated wood. Blue stain fungi grew mainly near the cross cut as well as in empty spaces such as micro-capillaries, parenchyma tissue or other wax free zones of treated wood.

Key words: Blue stain, hyphae, wax, wood.

INTRODUCTION

Blue stain in wood and timber is one of the defects that can cause serious economic losses in wood products (Thwaites et al. 2004; Chow & Obermajer 2007). Blue stain is characterised by a radial streaky stain of greyish black discoloration on the wood surface (Schmidt 1994). Such colouring of the hyphae is caused by the pigment melanin (Zink & Fengel 1989). Blue stain fungi feed on the contents of the living parenchyma cells (Weiß et al. 2000) and colonise sapwood exclusively (Schmidt 1994).

Various treatments with conventional biocides can impede a blue stain infection (Militz & Mai 2008). However, these wood preservation agents are controversial. Their use is limited by law because of health concerns, problematic waste disposal and their ecological toxicity (Pohlandt & Marutzky 1996; Kusian 1998; Guo et al. 2005, Chemikalienverbotsverordnung 2008). Several studies have been undertaken on biological control of sapstain fungi by using albino strains of different Ascomycetes. A review of albino Ophiostoma sp. fungal (Cartapip) application within the pulp and paper industry can be found in Farrell et al. (2005). The efficiency of colourless albino Ophiostoma sp. in maintaining the brightness levels of wood chips prior to pulping has been demonstrated for a number of softwoods and hardwoods. Chittenden and Singh (2009) found that the combination of chitosan and albino Trichoderma harzianum inhibited spore germination and hence colony formation of two sapstain fungi, Leptographium procerum and Sphaeropsis sapinea.

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An additional tool for improving the biological durability of wood, which meets the increased environmental awareness, is wood modification. Although limited, there has already been some research on blue stain in modified wood (Hill 2006). Heat treatment did not improve the resistance against blue stain (Aholá et al. 2002). Ewert and Scheiding (2005) reported a superficial blue stain infection of thermal treated timber. In contrast, Boonstra et al. (2007) reported absence of blue stain on heat treated radiata pine and Norway spruce. Oil heat treated Scots pine showed high resistance against blue stain (Petrič et al. 2006). Acetylated wood was infected by blue stain fungi (Beckers et al. 1994). Petrič et al. (2006) found a resistance of wood modified with DMDHEU (1,3-dimethylol-4,5-dihydroxyethyleneurea) against blue stain after 6 weeks of exposure. Also, Xie et al. (2008) detected reduced staining of wood modified with methylated DMDHEU after 18 months of weathering. Ritschkoff et al. (2003) found a prevention of blue stain in Scots pine treated with a siloxane formulation. Ghosh et al. (2009) noticed a strong resistance to blue stain attack in wood modified with a 10% amino-silicone macro-emulsion, both for pre-weathered and not pre-weathered wood. According to Treu et al. (2004), boiled linseed oil and maleinised oil decreased the blue stain infection of Scots pine sapwood, whereas paraffins had no effect.

The above investigations show contradicting results; however, there is a lack of information on the reasons for the inefficacy of some wood modifications. The objective of this paper was to monitor on a macroscopic and microscopic level the development of the blue stain fungus *Aureobasidium pullulans* in wax treated wood. The hydrophobic effect of wax (Borgin & Corbett 1970; Sell 1977; Scholz et al. 2009) and the physical blocking of the wood lumens by wax deposits were examined as influencing parameters for the penetration of blue stain hyphae within wood.

**MATERIALS AND METHODS**

**Wax treatment**

Blocks of Scots pine sapwood (*Pinus sylvestris* L.) and beech (*Fagus sylvatica* L.) with dimensions 350 × 50 × 10 mm (L × T × R) were impregnated at 100 °C in a vacuum (100 mbar, 1 h) and subsequently under pressure (12 bar, 2 h) with four different hot melting wax types: esterified montan acids (wax 1), modified plant wax (wax 2), amid wax based on 2-oxazolin (wax 3) provided by Clariant Produkte Deutschland GmbH (Gersthofen) and Fischer-Tropsch-paraffin (wax 4) from Sasol (Hamburg). Information on wax types is given in Table 1. The density of the treated and untreated wood samples

<table>
<thead>
<tr>
<th>Wax No.</th>
<th>Product name</th>
<th>Type</th>
<th>Total formula</th>
<th>Melting point (°C)</th>
<th>Viscosity at 100 °C (mPa s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Licowax E</td>
<td>Montan wax</td>
<td>C24–C34</td>
<td>81</td>
<td>27.9</td>
</tr>
<tr>
<td>2</td>
<td>Experimental</td>
<td>Modified plant wax</td>
<td>–</td>
<td>80–88</td>
<td>9.9</td>
</tr>
<tr>
<td>3</td>
<td>Experimental</td>
<td>Amid wax</td>
<td>C5H11NO5</td>
<td>77.5</td>
<td>10.8</td>
</tr>
<tr>
<td>4</td>
<td>Paraffint C80</td>
<td>Paraffin</td>
<td>C1nH2n+2</td>
<td>80–85</td>
<td>9.4</td>
</tr>
</tbody>
</table>
was determined according to DIN 52 182 (1976) standard. The moisture content was calculated under consideration of DIN 52 183 (1976). The volume of the samples was determined by measuring their dimensions.

**Blue stain test**

The blue stain test was carried out according to standard EN 152 (1988) by using *Aureobasidium pullulans* (de Bary) Arnaud, but with smaller samples (40 × 40 × 5 mm, L × T × R) and without pre-weathering. This fungus belongs to the tertiary blue stain type and favours moist timber (Schmidt 1994). It is known to have a rather complex life cycle involving yeast-like cells, hyphae, chlamydospores and intermediate forms (Kockova-Kratochvilova et al. 1980). Controls as well as wax impregnated samples were conditioned for 2 weeks at 20 °C and 65% relative humidity to reach moisture equilibrium. The samples were then sterilised by using gamma radiation (25 kGy, Isotron, Netherlands). The test took place in Kolle flasks filled with vermiculite over a period of 8 weeks. The samples were dried in a vacuum (0.1 bar) drying chamber at approx. 60 °C. The samples were evaluated for blue stain according to the ranking system of EN 152 standard from 0 to 3 (rank 0: no staining to rank 3 with 75% staining).

After the test, the moisture content of the samples was determined by drying at 50 °C and 0.05 bar in a vacuum-drying chamber. Additionally, the values of the wax treated specimens were compared to the dry wood mass without wax (average mass of the controls of the same sample group) because of a decrease of moisture due to the hydrophobic wax mass. It is advisable to calculate the moisture content on the basis of the dry wood substance (without wax), because the hydrophobic wax itself will not contribute to the uptake of water.

The samples were crosscut lengthwise in various positions to visually observe the penetration of fungal hyphae.

**Microscopic observation**

Radial and tangential microtome sections (20 µm thick) were obtained from the surface and up to 2 mm in depth for each specimen, stained with 1% safranin and mounted on glass slides in glycerin according to the usual methodology. Preliminary tests showed that safranin was satisfactory in staining the hyphae. Hyphae appeared brown-red and were easily traced through the wood elements. The sections were observed using a light microscope (Nikon eclipse E600) equipped with a digital camera (DXM 1200, Nikon Instruments Inc. NY, USA) that had the facility to embed scale bars in the images.

For scanning electron microscopy (SEM), wood blocks 5 × 5 × 5 mm (L × T × R) were taken from separate positions of each specimen. The blocks were split radially or tangentially with razor blades to obtain smaller samples (c. 3 mm³) with smooth radial and tangential surfaces. The small samples were mounted on stubs with carbon adhesive tape and coated with carbon using a sputter coater. Coated samples were observed with a scanning electron microscope (Supra 45, Leo Elektronenmikroskopie GmbH, Oberkochen, 5 kV).
RESULTS AND DISCUSSION

Blue stain

Based on the information on wax densities and the densities of samples after impregnation, wax uptake was satisfactory (Table 2). Table 2 indicates a valid blue stain test as the average moisture contents of infected specimens varied between 45–76% for Scots pine sapwood and between 42–59% for beech (moisture referred to the wood mass without wax). According to Schmidt (1994) the optimal moisture content for *Aureobasidium pullulans* ranges from 30 to 80%.

A rapid colonisation was noticed on the external surfaces of both the controls and the wax treated specimens after starting the test and over the subsequent weeks. Wax impregnation causes hydrophobation, so it is most likely that the average moisture content has a gradient from the inner wood (low) to the surface areas (high). As a result, almost all samples showed massive staining on the upper and lower surface. The evaluation according to the EN 152 standard showed staining of more than 75% on all but one sample surface, thus falling into rank 3. This could be explained by the higher moisture contents at the interface of wood and vermiculite (Ghosh *et al.* 2009). The only exception was noted for the lower surface of Scots pine sapwood treated with wax 2, which was evaluated as an early infection (rank 1). The virulent growth of *A. pullulans* showed with certainty that with hot melted wax, impregnated wood without biocides is not resistant against blue stain. This is not unexpected, because the waxes themselves do not have biocidal characteristics. For example from earlier research (Farrell *et al.* 2005; Schmidt 2006) it is known that natural wood waxes can be degraded by *Ophiostoma* sp. The use of waxes in combination with biocides is well documented in patent literature (US Patents 7297193, 4631302, *etc.*). This approach includes commercially available wax emulsion formulations and wax based antifouling coatings in which waxes serve as hydrophobic material and delay the leaching of the biocide components (Green & Schultz 2003).

The control samples of pine displayed an extensive visible infection on the cross sections while in the case of wax treated samples the infection was limited in principal close to surfaces in both Scots pine sapwood and beech. In some cases of wax im-

Table 2. Overview of the densities of waxes and wax treated wood and the moisture contents after the test.

<table>
<thead>
<tr>
<th>Type</th>
<th>Wax density (g/cm³)</th>
<th>Scots pine</th>
<th>European beech</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density (g/cm³)</td>
<td>Moisture¹ (%)</td>
<td>Moisture² (%)</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>0.52</td>
<td>76</td>
</tr>
<tr>
<td>Wax 1</td>
<td>1.02</td>
<td>0.90</td>
<td>31</td>
</tr>
<tr>
<td>Wax 2</td>
<td>0.96</td>
<td>0.96</td>
<td>28</td>
</tr>
<tr>
<td>Wax 3</td>
<td>0.99</td>
<td>1.06</td>
<td>21</td>
</tr>
<tr>
<td>Wax 4</td>
<td>0.90</td>
<td>0.95</td>
<td>23</td>
</tr>
</tbody>
</table>

¹) Moisture calculated on the basis of mass of wood and wax.
²) Moisture calculated on the basis of mass of wood without wax.
pregnated samples, mainly for Scots pine, the penetration of the ray cells was clearly visible. This was in accordance with Schmidt (1994), who describes a preferred hyphal migration of blue stain fungi through the ray cells and degradation of sugars, starches, proteins and fats within these cells. There is a strong colonisation on the surfaces in contact with the vermiculite. Once established, the hyphae start to expand through the ray cells but in the case of wax treated samples, the hyphae are unable to entirely penetrate the wood. This phenomenon might be due to the blocking effect of the wax deposits within the wood. A time-dependent superficial staining of modified wood (without biocides) could be explained by the storage substances within the spores. For basidiospores, Friese (1932) found 24% proteins and 3.7% lipids on average and minor nutrients such as iron or manganese contained within the dried matter. Joppien et al. (1972) examined the uredospores of the germ tube wall of *Puccinia graminis* and found 12.5% amino acids, 5.3% lipids and about 55% sugars. Also, the treated samples seem to be more infected on the surfaces than the controls. This difference can be explained by rapid fungal colonisation. The fungal activity takes place rapidly in the moist inner part of the porous untreated wood, especially within pine sapwood, instead of concentrating only on the surface as for the wax treated samples.

**Microscopic evaluation**

Characteristic microscopic images of fungal colonisation in wax treated wood are shown in Figures 1 and 2. There was considerable difficulty in preparation of sections due to the melting point of waxes (Table 1) and their solubility in organic solvents. Microscopic observations confirmed the occurrence of blue stain hyphae mainly close to the surfaces of the samples while a much smaller amount of wood tissue was colonised further away from the surface within the wax treated wood (up to 2 mm in depth). It was noticed that hyphae preferred the wax free wood tissue and were present in greater abundance in these parts (Fig. 1a, d; 2a, d). This could be explained by the mechanical barrier of the wax deposits and their influence on the moisture content. Scholz et al. (2010) showed the presence of wax within open pits in wax treated pine. Furthermore, not every micro-capillary could offer enough space for a rapid penetration of the relatively thick blue stain hyphae. Moreover the wax treated specimens

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**Figure 1.** Microscopic images of pine sapwood impregnated with different wax types. – a: Tangential section showing hyphae in wax free tracheids and within the ray parenchyma (wax 1). – b: Radial section: hyphae between wax column and cell wall (wax 2). – c: Radial section: hyphae inside ray parenchyma but not in ray tracheids (wax 4). – d: Tangential section: fungi hyphae within the empty tracheids (wax 3).

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**Figure 2.** Microscopic pictures of wax impregnated beech. – a: Tangential section: hyphae in wax free vessels (wax 1). – b: Radial section showing hyphae in the wax free ray and parenchyma cells as well as in wax cracks (wax 2). – c: Radial section: hyphae across cracks of deposited wax in vessels (wax 1). – d: Hyphae with chlamydospores (arrow) behind wax fragments (wax 1).
have a lower moisture content (Table 2) which indicates the hydrophobic effect of wax deposits. According to Schmidt (1994) blue stain hyphae penetrate the wood radially through the rays. Thin hyphae grow through the margo and thicker ones push the tori aside. These observations were also confirmed for wax treated wood (Fig. 1c; 2b).

Blue stain hyphae grow through the ray parenchyma, where the food source is located, in both Scots pine and beech (Fig. 1a, c; 2b). In accordance with this observation, the ray parenchyma cells were free of wax deposits. This was also reported previously for Scots pine and beech (Scholz et al. 2010).

It should be noted that no hyphae were observed in the ray tracheids of Scots pine (Fig. 1a, c). In fact the hyphae used the wax free ray parenchyma tissue in both pine and beech, wax free axial tracheids in pine (Fig. 1a, d) and vessels in beech (Fig. 2a, d) as well as minor capillaries between cell walls and wax (e.g. Fig. 1b; 2c), fine cracks and air void spaces within the wax deposits, as preferred pathways for their migration within wax impregnated wood. Such capillaries, cracks and void spaces were created by the shrinking of the congealing wax after impregnation or by poorly impregnated wood (Scholz et al. 2010).

Variations between the different wax treatments could not be confirmed on a microscopic level. It could be shown that neither the different chemical substitution of the chosen waxes nor the different shrinkage behaviour influenced the performance of the treated wood. The thin microscopic sections (20 µm) cause a release of wax deposits which are not chemically bound to the wood cell wall.

The question is whether hyphae are able to penetrate wax or not. Liese (1970) reported the possibility of cell wall perforation by transpressorium hyphae. It has also been found that blue stain fungi are able to penetrate coatings and paint films by enzymatic and/or mechanical means (Winters et al. 1978; Sharpe & Dickinson 1992; Bardage & Daniel 1997; Van den Bulcke et al. 2007). These capabilities of blue stain fungi could also be shown for penetration of wax columns. In some light microscopic views, fungi possibly seemed to penetrate the wax (e.g. Fig. 1a, c; 2b, c). Such a fact could not be definitely confirmed due to the transparency of wax and overlap effects. Closer inspection under SEM did not show any evidence of wax penetration by hyphae. It seemed that blue stain hyphae prefer to use the void spaces and wax capillaries for their migration rather than penetrating directly through the wax. However, the outdoor use of wax treated wood seems impractical without addition of biocides to maintain decorative surfaces.

CONCLUSIONS

Wood impregnated with hot melting and biocide free waxes shows an intensive infection by blue stain on the surface. However, in contrast to the untreated controls, the hyphae are mainly limited to the surface. The hyphae penetration in wood cannot be impeded by the blocking of wax deposits due to remaining micro-capillaries between the cell wall and congealed or wax free tissue such as parenchyma cells. The influence of the hydrophobic wax within the wood lumens is more important in relation to the decrease of moisture content from the outer to the inner wood parts.
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REFERENCES


