Growth of Mold and Rot Fungi on Copper-impregnated Scots Pine Sapwood: Influence of Planing Depth and Inoculation Pattern

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The biocidal properties of an industrially used copper-based preservative were evaluated at different planing depths on exposure of pine wood to mold fungi in direct and indirect contamination methods, with simultaneous verification of white rot fungi virulence on wood. The preservative was an aqueous solution of copper carbonate, 2-aminoethanol, and quaternary ammonium compounds. Full cell preservative impregnation efficiency against visual mold fungi growth was tested on sapwood surfaces planed to different depths before impregnation. The virulence of two white rot fungal strains of *Trametes versicolor* (441 and JPEI) against the dried non-impregnated and impregnated wood samples was also tested. The unplaned surface of impregnated timber was occupied by air-borne contaminants, such as *Paecilomyces variotti* and *Aspergillus niger* up to 30%, and, even after impregnation it was necessary to process the surface to avoid micro-fungi settlement. The virulence of the tested rot fungi strains was confirmed by the aggressive degradation of non-impregnated wood with a mass loss of over 40%. Both *Trametes* sp. strains degraded the preservative-impregnated wood with a mass loss of 3.1% to 4.8%, but degradation by the JPEI strain was more intensive and more dependent on planing depth than the other strain (441).

Keywords: Scots pine sapwood; Impregnation; Planing depth; Mold; Rot; Fungi; Test

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INTRODUCTION

The biodegradation of wood is a critical factor in determining its performance, especially for wood exposed to humid and wet conditions in both indoor and outdoor environments. To protect against biological damage, a large number of preservation methods for wood products have been developed and used. For instance, the impregnation of wood using copper-salt solutions has been used to prevent biodeterioration since 1718 (Unger *et al.* 2001). Today, copper salts are the dominant preservative for wood products, but compounds, such as chromated copper arsenate (CCA), are not allowed in Europe and the USA due to their environmental toxicity. The leaching of copper ions from impregnated wood can lead to various health disorders and accumulation in aquatic species (Peres and Pihan 1991; Tchounwou *et al.* 2012). Therefore, researchers and the saw-mill industry are trying to develop safer combinations and other solutions for wood impregnation with copper-containing chemicals to reduce environmental risk (ECHA 2011). One method of reducing the copper ion concentration in impregnating liquid is the effective combination of organic and biodegradable biocides.

The biocidal action of copper-based treatments could be explained by separate effects of hydro-peroxide radical formation due to the reaction of hydrogen peroxide and copper ions, binding to the cell membranes, and decreasing membrane fluidity (Cervantes

and Gutierrez-Corona 1994; Suwalsky *et al.* 1998; Borkow and Gabbay 2005). However, some fungi have developed systems to overcome the biocidal action of copper-salt preservatives. The general mechanisms of fungal resistance to heavy metals proposed by Nies (1999) suggest decreasing the concentration of heavy metals in wood *via* effluxion of such compounds, resulting in isolation by creating complex compounds or reduction to a less toxic (oxidation) state. After several years of research, it was found that such a treatment is only partially effective with soft rots, whereas brown rot fungi *Fibroporia radiculosa* can detoxify copper compounds by forming oxalates (Ohno *et al.* 2015). As a part of global geochemical processes, fungi can leach, recover, detoxify, and remediate metal compounds (Gadd 2017).

Primary wood-colonizing fungi are a number of ascomycetes (deuteromycetes) and basidiomycetes, which are initially transmitted by air to wood surfaces. The ascomycetes on wood decompose mainly cellulosic fractions when basidiomycetes or rot fungi decompose lignino-cellulosic matrix of wood (Crous *et al.* 2018). The spores germinate under favorable moisture conditions in the presence of low-molecular nutrients. Rot fungi usually invade wood at later stages of decomposition from soil or insect contamination (Zabel and Morrell 1992). The diversity of fungi is enormous, but mold fungi associated with bio-based building materials are restricted to air-borne, xerotolerant, or xerophilic types. Most of these fungi are also clinically important, as they are known to be mycotoxin producers (Samson and Houbraken 2010) and can cause substantial deterioration in indoor environments. The physical and chemical properties of wood surfaces and the surrounding humidity are the main factors that determine the suitability of conditions for fungal development. Wood surface conditions are influenced by the migration of extractives towards the surface during the drying process and enrichment of the surface by soluble nutrients required for the growth of microorganisms (Terziev and Nilsson 2005; Terziev and Boutelje 2007; Terziev 2009).

Pressure impregnation, like the full-cell, Lowry, and Rüping methods, is the most common way to treat pine sapwood boards with solutions of copper salts (Reinprecht 2016). During impregnation the preservative penetrates into the wood *via* various pore systems (Ahmed *et al.* 2012) and is assisted by a pressure gradient of between 10 bars and 15 bars. The copper-salt complexes are also believed to be able to move into the cell wall of wood through a diffusion process. The ability of copper ions and ethanolamine to form strong interactions with phenols and carboxylic acids in the wood stabilizes the structure (Ruddick *et al.* 2005; Zhang and Kamdem 2007). However, the impregnation process sometimes fails when there is an uneven distribution of chemicals in the cell structure due to the morphological nature of wood, the extractives, and the relocation of the extractives during the drying process before impregnation as was studied by using histochemical methods (Ahmed *et al.* 2012).

Various test methods have been designed to evaluate bio-based building materials with respect to their vulnerability to fungal damage in various applications. The reliability and variation within the results of such tests are discussed widely, especially when evaluating mold tests (Kutnik *et al.* 2014). The majority of studies of vulnerability of building material to fungal growth focused on influence of temperature and relative humidity in different combinations but not to properties of material itself and relevant processing technologies such as drying and planning. The hypothesis proposed in this work is that by removal of wood surface material enriched with migrated nutrients together with impregnated biocides it is possible to lower fungal growth and increase durability of wood material. Therefore, this study aims to explore the impregnation efficiency by studies on

the biocidal properties of industrially used copper-based preservative treated wood at different planing depths during the exposure to mold and white rot fungi. The influence of different test methods were also performed.

EXPERIMENTAL

Materials

Wood samples

A total of 42 center-sawn Scots pine (*Pinus sylvestris* L.) boards (50 mm × 125 mm) with the maximum possible sapwood ratio were selected directly after sawing in a Kåge sawmill in Västerbotten county in northern Sweden. Each board was further divided from the top end into up to four 1-m-long samples in order to enclose the heartwood on the upper flat side. In addition, 50-mm-long pieces were taken each sample to determine the average raw moisture content (MC) (oven-dry method) and average density $\rho_{0,raw.}$ A total of 96 samples, end-sealed with Sikaflex 221 (Sika Sverige AB, Spånga, Sweden), were randomly distributed into four equivalent batches that were dried and conditioned in two identical series in a small-scale laboratory kiln, using an industrial schedule with a target MC of 18%. The drying schedule used in the experiments is shown in Fig 1.

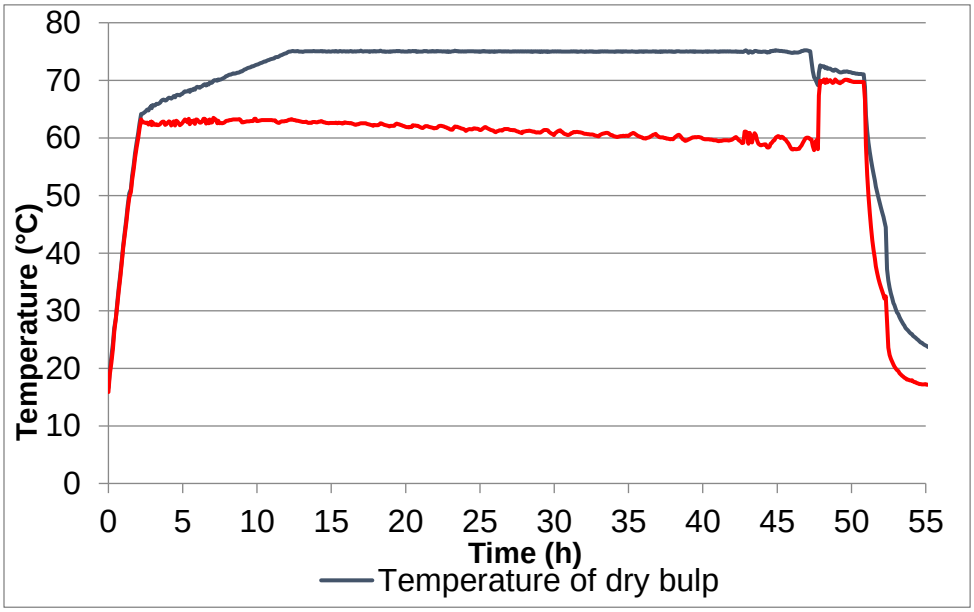
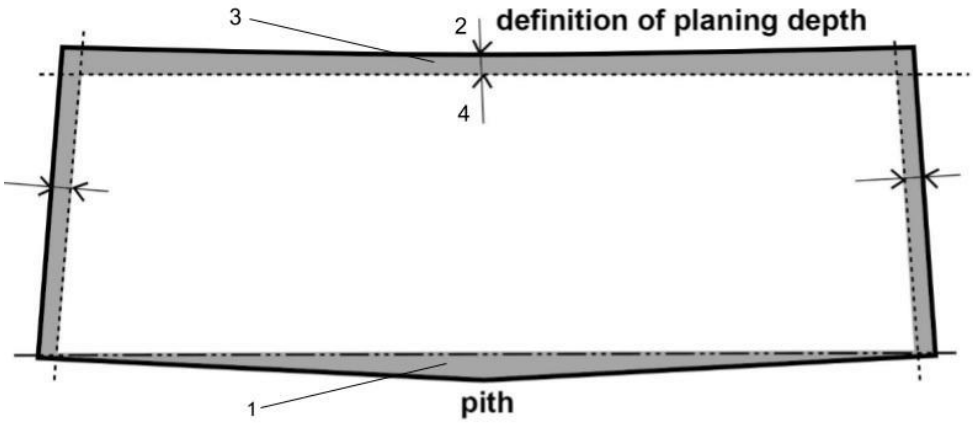


Fig. 1. Drying schedule with a 3 h conditioning stage

After drying, the specimens were conditioned for 3 h and cooled. The MC of each sample was measured using an electrical moisture meter. The four batches were then randomly selected for machine setting planing depths of 0 mm, 0.7 mm, 2.0 mm, and 4.0 mm. The planing depths were measured with a caliper for each specimen before and after planing. Planing depth, taking failure of cross-section into account, is defined in Fig. 2.



1) **planing of pith side**
2) **thickness measurement immediately above the pith**
3) **planing of the sapwood side**
4) **thickness measurement immediately above the pith**

Fig. 2. Planing schematic and definition of planing depth with cupping of cross-section

Methods

Impregnation and evaluation of impregnation results

After all the samples were weighed, the batches were transported to the impregnation plant where the four batches were impregnated with copper salt solutions in one run in an industrial autoclave. The impregnating agent was Celcure AC 800 (copper carbonate 17.3%, 2-aminoethanol 35%, *N*-alkyl(C12-16)-*N*-benzyl-*N*,*N*-dimethyl-ammonium chlorides 4.8%, and water) supplied by Koppers in Helsingborg, Sweden. The impregnating agent was diluted with water to a concentration of 3.4% and a liquid temperature of 15 °C.

Impregnation was performed according to the current standards of NTR class AB (Nordic Wood Preservation Council 2012) to meet the product requirements for industrially protected wood according to the Nordic Wood Preservation Council, which requires full sapwood penetration in Scots pine. The process included the following stages: 1) Wood material placed in an autoclave was pre-vacuumed to 90% for 20 min; 2) Preservative fluid was inserted and an overpressure of 1400 kPa was maintained for 40 min; and 3) The material was vacuumed for 20 min.

The subsequent fixation/drying that is usually included in industrial production was not performed in this case. Immediately after impregnation, the samples were transported to the laboratory, where each sample was weighed to record the impregnation agent uptake, which was calculated as the wet weight increase per unit volume.

Each sample was then cut into four parts, with as few knots in the cross-section as possible and then stacked on top of each other with all of the cross-sections turned so that they were approximately 25 cm from the surface (Fig. 3). After stacking, all the cross-sections were brushed with a heartwood-sensitive reagent to obtain a distinct boundary between the softwood and the heartwood. The impregnated sapwood was identifed as the green-colored areas, the heartwood was identified as the reddish areas, and impregnation failure was identified as the wood-colored areas. The heartwood proportion was calculated, and the impregnation agent percentage uptake in the sapwood was estimated according to

the proportion of cross-cut area of sapwood to the uptake of impregnation agent, with the assumption that there was no uptake of impregnation fluid in the heartwood.,

a) b)

Fig. 3. a) Batches immediately after impregnation; b) Examples of cross-sections of individual specimens divided into 1-m lengths, showing impregnation results and heartwood dyed with a heartwood reagent

Fungal test

The specimens for the mold fungi test included only knot-free sapwood parts taken from the upper sapwood side and cut into two replicates with a size of approximately 110 mm × 60 mm × 10 mm. The replicates were suspended in plastic holders in two separate plastic boxes with dimensions of 600 mm × 300 mm × 350 mm with the open wall sealed *via* an air-transparent polymeric membrane, as shown in Fig. 4. The climate in each box with indirect inoculation was T= 21.1 ± 0.1 °C and RH = 90 ± 1%, for each box with direct innoculation climate was T = 22.1 ± 0.1 °C and RH = 89 ± 5%.

The four mold fungi of *Penicillium commune* Thom, C., *Aspergillus niger* van Tieghem, *Paecilomyces variotii* Bainier, and *Mucor plumbeus* Bonord. (Culture collection, Wood Science and Engineering, Luleå University of Technology) cultivated on malt-extract agar (Merck, Darmstadt, Germany) were used for fungal inoculation. Two methods of inoculation were used, including the indirect method using open petri plates with pure cultures placed on the bottom of the box according to ASTM D7855/D7855M - 13 (2013), and a direct method where a suspension of the four fungi in a concentration of 10^6 spore/mL, prepared according to SS-EN 15457:2014 (2014), was sprayed directly onto the wood surface. To evaluate the influence of MC, the initial MC and final equilibrium MC were recorded. Images of mold growth on the surface were obtained *via* digital photography by WolfVision VZ-8light[4], and the growth was assessed in two ways using ImageJ software (National Institute of Mental Health, version 1.51j8, Bethesda, Maryland, USA). In particular, growth was assessed as the percentage of the total area of the board that was fungi and as the covered area of fungal growth (Schindelin *et al.* 2012). The individual mold species on the wood surfaces were identified with the naked eye and an optical microscope to identify the morphological characteristics of each fungi (Samson and Houbraken 2010).

a) b)

Fig. 4. Mold growth tests: a) indirect and b) direct methods

Two white rot strains (441 and JPEI) of *Trametes versicolor* (L.) Lloyd were obtained from the Aloha Medicinals Mushroom Culture Bank in Carson City, NV, USA. All the cultures were stored on solidified malt extract agar at 4 °C ± 1 °C with visual control of their purity and activity. The virulence of each fungal strain was assessed by the mass loss of the treated and non-treated wood samples, determined according to the SS-EN 113 (1996) standard with each sample having dimensions of 30 mm × 15 mm × 5 mm. The experimental design and test methods are explained in the Table 1.

Table 1. Experimental Design

Experiment	Test method	Number of replicates at planning depth			
		Group 1 (0 mm)	**Group 2** (0.7 mm)	**Group 3** (2.3 mm)	**Group 4** (4.0 mm)
Mould test	**ASTM D7855/D7855M-13**	5	5	5	5
	SS-EN 15457:2014	5	5	5	5
Rot test	**SS-EN 113** *Trametes versicolor 441*	20	20	20	20
	SS-EN 113 *Trametes versicolor JPEI*	20	20	20	20

A complete data matrix for the mold test comprising of totally 40 observations (2 methods × 4 groups × 5 replicates) was obtained for each treatment method (indirect and direct contamination) and each group (planing depth).

The analysis of the white rot test included a data matrix for the dry mass loss from totally 200 observations (2 fungi × 5 batches × 20 replicates). A univariate analysis was performed to study the influence of the planing depth on fungal growth after impregnation by comparing their mean differences using IBM SPSS Statistics 20.0 (Armonk, NY, USA) software. This approach makes it possible to summarize and compare differences across one or more factors, or categorical variables.

RESULTS AND DISCUSSION

Penetration of Preservation Solution

This experiment was designed to evaluate fungal infestation on dried and impregnated wood. Samples planed to a depth of 4 mm (group 4) had the highest uptake of preservative solution (Table 1). In the wood that was not planed before impregnation (group 1), the amount of preservative solution in the wood after impregnation was 9% less than that of group 4, which had greater planing depth. (Table 2). No statistically significant difference in the uptake of impregnation solution was observed between the samples planed to depths of 0.7 mm and 2.3 mm, which could be of interest for the many sawmills that are striving to achieve lower planing depths. Even though the heartwood content was slightly higher and the density statistically significant higher for the boards in group 3 than for the boards in other groups, no influence on boards uptake of impregnation solution was observed.

Table 2. Scots Pine Sapwood Properties and Uptake of Preservation Solution During Impregnation for Groups with Different Planning Depth

Parameter	Group 1	Group 2	Group 3	Group 4
Planing depth (mm)	0[a]	0.7[a]	2.3[a]	4.0[a]
Heartwood content (%)	39	39	41	38
Density (kg/m^3) Green Dried	 813[a] 393[a]	 804[a] 386[a]	 768[a] 402[a]	 812[a] 372[a]
Moisture content (%) Green Dried	 107.7[a] 17.5	 108.9[a] 17.8	 91.7[a] 19.0	 118.8[a] 18.0
Impregnation uptake in sapwood (kg/m^3)	584	615a	626	643
[a] - Significant mean difference between groups ($p < 0.05$)				

The penetration of impregnation agents starts from the surface of material, and the properties of the surface are vitally important for impregnation retention (Terziev 2005). The design of the impregnation agent used in this experiment allows ethanolamine to increase the solubility of copper salts in the impregnation liquid (Forest Products Laboratory 2010) and thereby penetrate deeper into the wood. Penetration is believed to take place through membranes in various pits into the wood, and the authors' results demonstrated the influence of the surface of the material on impregnation agent retention.

Fungal Test

The fungal test was designed to be similar to the primary colonization of molds. To mimic such an environment, the authors applied fungal species (*Penicillium* sp., *Aspergillus* sp., *Paecilomyces* sp., and *Mucor* sp.) tolerant to dry conditions that had previously been isolated from dried wood. This made it possible to identify fungal colonization during the infection process in both the wet and natural states of innoculation. The determination of mean mold area at a similar initial MC of the boards revealed the importance of both the method of inoculation and the planing depth (Table 3). The moisture

content (MC) of the samples with direct inoculation was higher in 10% in average than the MC after natural airborne inoculation, which was probably due to the higher climate fluctuations. No difference in the initial MC was detected between the groups for the different mold inoculation methods (Table 2).

Table 3. Mean of Mold Areas and MC of Scots Pine Sapwood Boards Treated by Different Fungal Inoculation Methods

Sample Group No.	Mold Area (%)	Mold Area (mm^2)	MC Before Test (%)	MC After Test (%)
Indirect Fungal Inoculation				
1	32.4[a]	2222.8[a]	9.6	20.0
2	0[a]	0[a]	9.5	17.4
3	0[a]	0[a]	9.5	20.5
4	0[a]	0[a]	9.6	20.5
Direct Fungal Inoculation				
1	24.5[a]	1659.01[a]	9.6	21.8
2	0.4[a]	25.9[a]	9.7	21.8
3	0.1[a]	3.5[a]	9.6	21.4
4	0[a]	2.4[a]	9.6	22.1
[a] - Significant mean difference between the groups ($p < 0.05$)				

Figure 5 shows the colonization patterns of the boards with both inoculation methods. The fungal colonies growing on the wood surface were different. The *Paecilomyces variotti* and to a smaller extent the *Aspergillus niger* were dominating other species *via* indirect contamination. However, after spraying the molds, most of the wood surface was occupied by *A. niger*. Perhaps *P. variotti*, which produces conidia as chains that easily become airborne, would be better equipped for a more natural infection, whereas *A. niger* conidia forms a single dry spore and functions better in an excess of water (Adan and Samson 2011).

The tendency to produce more aerial mycelia at the lower edges of boards was observed in the natural contamination method (see red arrow in Fig. 5). The authors suggest that the fungi here initiated more tissue development to absorb water vapor from the surrounding atmosphere (Pypker *et al.* 2017). Alternatively, the fungi might initiate aerial morphogenesis to produce more reproduction tissue for further survival and new habitat colonization under extreme conditions, such as low water content environment (Van Laarhoven *et al.* 2016; Segers *et al.* 2017).

Thus, it was evident that the application method influenced the susceptibility of the wood materials to mold attack, which was in line with previous studies on gypsum (Bekker *et al.* 2012). The authors confirmed that the planing depth influenced the mold growth development even in the preservative-treated wood (see Table 2). The addition of water to the wood samples during spraying increased the amount of water and stimulate fungal growth, especially when the wood moisture content stays below the fiber saturation point and the surrounding atmosphere contains a large amount of water vapor. That was similar to the finding of Van Laarhoven *et al.* (2015) that water content stimulates early colonization and the hyphal extension rate of mold fungi. The dominance of the fungus *A. niger* over other isolates could be explained by its ability to adhere more strongly to the wood surface than other anamorphic fungal species (El Abed *et al.* 2010). To the authors' knowledge, the growth of fungal species on wood surfaces of *Mucor plumbeus* has not been discovered after either indirect or direct inoculation.

Fig. 5. Surface appearance of samples inoculated by the direct and indirect inoculation methods. The red arrow highlights the aerial mycelia.

This study is the first to show the differences in contamination of impregnated wooden surfaces by common indoor fungi. To some extent, the planing depth and planing itself influenced mold growth (Terziev 2009; Sehlstedt-Persson *et al.* 2016; Johansson *et al.* 2017). Particular attention must be given to the indirect method of testing, where medically essential *Paecilomyces variotti* showed a dominant coverage of the wood surface. The rougher unplaned surface in group 1 contained more microvoids with water, which were favorable for spore germination (Johansson *et al.* 2017). Molds were observed only after the direct inoculation of planed wood surfaces, and mainly on the wood surface closer to the original wood surface than deeper into the material. However, further study is necessary to clarify the availability of nutrients for filament germination, hyphal growth,

and sporulation on the surface. The authors' previous study revealed that mold fungi occupied only approximately 100 µm of the wood surface in depth and that the fungal growth was associated with the migration of nutrients during wood drying (Sehlstedt-Persson *et al.* 2011; Ahmed *et al.* 2013). However, limited information has been presented about fungal community development on the wood surface. For example, in the authors' past study, all three fungi had the same spore size (Samson and Houbraken 2010).

A much lower colonization was observed in the planed than unplaned surfaces (Table 3). Enrichment of nutrients takes place during artificial drying (Terziev *et al.* 2009); however still they are present in a dried wood deeper than at 0.7 mm. It is possible that water-soluble nutrients are dissolved and migrate along with the impregnating solution into the wood during impregnation treatment changing the gradients of nutrients formed in the wood during the wood drying. Surface roughness could also be of importance since indirect inoculation colonized the planed wood surface less than direct (even though the unplaned surface showed more colonization for indirect than direct inoculations).

The white rot fungus (*Trametes versicolor*) is an essential fungus for testing the resistance of preservative-treated wood material to biodegradation according to EN113 (1996). The virulence of the two strains of white rot fungus (*Trametes versicolor*) on impregnated and non impregnated samples was evaluated after 16 weeks of exposure (Table 3). Those results may also be of importance for further use in biodegradation assays. The general rule in the assessment of new strains is that the mass loss should be greater than 20%, according to SS-EN 113 (1996). During the trial, the fungi demonstrated aggressive behavior, and there was a mass loss of approximately 44% for strain JPEI and 41% for strain 441 in the wood samples without impregnation, which met the criteria stated above (Table 3).

Table 4. Mean Mass Loss of Scots Pine Sapwood Boards Treated with Two Strains of *Trametes versicolor* According to SS-EN 113 (1996)

Parameter	Mass loss (%)
Strain 441	
Group 1 (planning depth 0 mm)	4.4[a]
Group 2 (planning depth 0.7 mm)	3.7[a]
Group 3 (planning depth 2.3 mm)	3.4[a]
Group 4 (planning depth 4.0 mm)	4.2[a]
Control (without impregnation)	40.6[a]
Strain JPEI	
Group 1 (planning depth 0 mm)	4.8[ab]
Group 2 (planning depth 0.7 mm)	3.3[ab]
Group 3 (planning depth 2.3 mm)	3.1[ab]
Group 4 (planning depth 4.0 mm)	3.6[ab]
Control (without impregnation)	43.9[a]
[a] - Significant mean difference between impregnated groups and control (without impregnation), $p < 0.05$; [b] - The significant mean difference between impregnated groups of samples, $p < 0.05$	

The mass loss of impregnated samples treated with strain 441 showed a significant difference from that of the control samples without the impregnation agent, but there was no significant difference between the impregnated batches with different planing depths.

For samples degraded by strain JPEI, significant mean differences were found between the samples also treated with impregnation (Table 4).

White rot fungus (*T. versicolor*) is a selective degrader of lignocellulosic material, particularly in the early stages of decay (Shirkavand *et al.* 2017). The primary target of the attack is hemicellulose, but lignin and cellulose are also degraded (Chen *et al.* 2017). The effect of planing depth on mass loss is shown in Table 3. The mass loss was lower at a deeper planing depth for strain JPEI, but not for 441. The difference in the extent of degradation of wood by the two white rot fungi may have been due to phylogenetic variability within the species. Intraspecies variability in genetic and phenological traits are evident in the fungal kingdom and can cause fungicide failure (Georgopoulos and Skylakakis 1986; Nilsson *et al.* 2008). The agar media in the bottles developed a greenish color at the end of the test. It was possible that the microorganisms assisted the leaching of copper from the impregnated wood samples, which may be a result of a detoxification mechanism *via* copper-transporting ATPase pumps (Weissman *et al.* 2000; Hall 2002; Hastrup *et al.* 2005; Ohno *et al.* 2015).

CONCLUSIONS

1. The mold fungal damage of copper-impregnated Scots pine sapwood was influenced to a greater extent by the planing depth compared to the infection method.
2. The unplaned surface of impregnated timber can be occupied by air-borne contaminants, such as *Paecilomyces variotti* and *Aspergillus niger*, up to 30%, but the extent of mold coverage was much less on the planed impregnated timber. Even after impregnation by toxic compounds, the wood surface required processing to avoid or hinder micro-fungi settlement.
3. The new strains of white rot fungus (*Trametes versicolor*) quite aggressively degraded the non-impregnated wood with a mass loss of over 40%.
4. Both strains of white rot fungus (*T. versicolor*) degraded the wood impregnated with an industrially used copper-based preservative, with an estimated mass loss of 3% to 5%. One of the strains (JPEI) was more incoherent and sensitive to planing depth.

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