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Does copper tolerance provide a competitive advantage for degrading copper treated wood by soft rot fungi?

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ABSTRACT

The ability of soft rot fungi possessing strong (*Phialophora malorum*), medium (*Phialophora mutabilis*) and poor copper tolerance (*Chaetomium globosum*) to degrade untreated and $CuSO_4$ and micronized copper treated birch- and pine wood was assessed using ENV 807 standard tests. The aim was to determine whether an ability to grow on Cu-agar and copper in liquid cultures can be transcribed into a competitive advantage to degrade Cu-treated wood. An ability to tolerate high copper levels *in-vitro* was not correlated with increased decay by the fungi but rather reflected the native chemistry of the wood cell walls. *Both untreated and Cu-treated wood were degraded by the three fungi and showed aggressiveness in the order C. globosum > P. mutabilis > P. malorum* Higher mass loss was recorded for birch than pine and decreased progressively as the copper loadings increased with *statistically* insignificant difference noted between Cu-treatments. Microscopy showed decay at the cell wall level to reflect degree of lignification with parenchyma cells degraded first in both untreated and Cu-treated wood. Results indicate presence of copper and its toxicity is unlikely to be the main reason for preventing soft rot decay of wood but rather the additive effect of copper binding to the wood material.

1. Introduction

The biomineralization of wood in-service represents a major economic loss to society throughout the world. Traditionally, wood has been protected by the use of a variety of broad-spectrum (toxic) wood preservatives based on heavy metals and arsenic (e.g. Cu-Cr-As; (CCA)). While preservatives containing As are now prohibited in both above and in-ground situations in Europe and USA, they still remain the treatments of choice on the world market. Fundamentally, the protection of wood is based on inhibiting fungal decay of substrates by keeping the wood dry, preventing growth through toxicity, and/or preventing attack of the substrate through some type of substrate modification (e.g. binding metals or chemical modification of wood components). Over the last decade several more environmentally acceptable preservative formulations based on true chemical wood modifications (e.g. acetylation, furfurylation) or impregnation with oils and silicones have been designed and marketed. However, despite this development, Cu-based (e.g. Tanalith, Wolmanit, CCP, CC, alkaline copper quarternary (ACQ)) preservatives are currently and likely to remain in the near future the major form of in-service protection worldwide. This is perhaps further emphasized by the introduction of micronized-Cu (MC) in USA since 2006, which after only a few years took 80% of the market for the protection of wood in ground contact situations. Wood in natural environments maybe degraded by a variety of decay fungi causing either white, brown or soft rot (Blanchette et al., 1989; Daniel, 2014, 2016). Brown- and soft rot fungi are generally recognized as the most important economically in the biodegradation of preservative treated wood in-service both in- and above ground contact situations (Arantes and Goodell, 2014; Daniel, 2014).

It is widely recognized that wood treated with copper-based chemicals can often show reduced service life through aggressive decay by wood rotting fungi (e.g. Jermer, J., 2004; Freeman and McIntyre, 2008). In the majority of reported cases, decay is caused by brown rot fungi, several species of which are reported as copper tolerant with possibly the best known being *Antrodia vallantiii* (Sutter et al., 1982; Stephan, 1994; Råberg and Daniel, 2009). Other brown rot species include various *Antrodia radiculosa* species and strains, several of which also show tolerance to other metals including chromium and arsenic (Clausen et al., 2000; Jenkins et al., 2014; Green et al., 1991; Green and Clausen, 2003). The exact

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biochemical mechanisms used by brown rot fungi for degrading copper treated wood are still poorly understood but are thought to involve a simultaneous ability to both depolymerize cellulose rapidly and at the same time deactivate (i.e. bind-up) or detoxify free copper). In contrast to brown fungi, soft rot fungi are not known to cause rapid decay of wood or depolymerization of cellulose but can show high copper tolerance with some species/strains growing on 10% (w/w) copper sulphate supplemented agar (Daniel and Nilsson, 1988; Karunasekera and Daniel, 2013), a level much higher than that known for any brown rot fungi apart from *A. vallantii* (Sutter et al., 1982). The mechanism of wood decay by soft rot fungi after colonization is well known through the development of characteristic cavities aligned with cellulose microfibrils within the secondary cell walls of wood cells (i.e. Type I) or through erosion of cell walls from the lumen (i.e. Type II) (Daniel and Nilsson, 1998; Daniel, 2014, 2016). Previous electron microscopy and X-ray microanalytical studies have shown the ability of soft rot fungi (e.g. *Phialophora mutabilis*) to bind copper indiscriminately within cavities both extra- and intracellularly and for hyphae to penetrate high levels of copper precipitated on the cell lumina of fibres (Daniel and Nilsson, 1989). The true mechanism of copper detoxification is however unknown. While copper tolerant soft rot fungi are known to degrade copper-treated wood, either as the sole metal or in combination with other metals (e.g. in CCA, CCP), it is not known whether this ability can provide a competitive advantage although copper tolerant fungi are frequently the major fungal groups isolated from copper-treated wood from in-service situations (Henningsson and Nilsson, 1971; Nilsson and Henningsson, 1978; Råberg et al., 2014).

The purpose of the present study was to test whether the ability of copper tolerant soft rot fungi to grow on copper-supplemented agar *in-vitro* provides an added ability to degrade copper treated wood (as $CuSO_4$ or micronized copper (MC)) with commercial loadings under strict test conditions over longer periods of time (i.e. 6 and 9 months). Three well known soft rot fungi were used: *Phialophora malorum*, a highly copper tolerant fungus; *Phialophora mutabilis* a medium copper tolerant fungus and a strain of *Chaetomium globosum* found previously as only weakly copper tolerant (Daniel and Nilsson, 1988). Previous studies showed both *P. malorum* and *P. mutabilis* to tolerate and grow at far higher copper levels than the toxic threshold reported for soft rot in both birch (0.39% Cu w/w) and pine (0.07% Cu w/w) (Butcher and Nilsson, 1982; Daniel and Nilsson, 1988).

2. Materials and methods

2.1. Fungal strains

The three fungal strains (Table 1) were obtained from the culture collection maintained at the Department of Forest Products/Wood Science. Cultures are routinely maintained on 2.5% w/v malt extract agar (MEA) in Petri dishes and grown at 20 °C. *Phialophora malorum* [M. N. Kidd & A. Beaumont] McColloch has been shown in previous studies to possess high copper tolerance on $CuSO_4$ supplemented agar and in liquid cultures (Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988; Karunasekera and Daniel, 2013). *P. mutabilis* [J. F. H. Beyma] Schol-Schwarz [1970] (=*Lecythophora mutabilis*) has been shown under similar Cu-agar and Cu-liquid culture conditions to be mildly copper tolerant. *Chaetomium globosum* (Kunze: Fries, Telemorph) (strain F-171-1, ATCC 34152)) (syn = *Chaetomidium japonicum)* shows limited copper tolerance (Daniel and Nilsson, 1988) and is considered poorly copper tolerant. The *Phialophora* strains were originally isolated from copper-treated wood (poles and stakes) in service (Henningsson and Nilsson, 1976) (Table 1) and their molecular identification recently confirmed (Karunasekera and Daniel, 2013).

2.2. Wood samples, copper impregnation and fungal inoculation

Copper sulphate ($CuSO_4 \times 5H_2O$) (VWR, Sweden) and an emulsion of micronized copper (MC) particles was diluted in deionised water to produce formulations containing 0.2, 0.4 and 0.6% w/w (pure) copper. Scots pine (*Pinus sylvestris* L.) sapwood and birch (*Betula verrucosa* Ehrh.) samples (5 × 15 × 40 mm; along the grain) were impregnated by the full-cell method (200 mbar for 20 min followed by 6 bar pressure for 90 min). Untreated samples were used as controls. After drying and conditioning at room climate, wood samples were immersed in deionised water for 10 min prior to exposure in the test to increase the moisture content to encourage fungal colonization. The wood blocks were not leached and thereby retained both fixed and non-fixed copper.

Treated and untreated samples were exposed to the soft rot fungi (Table 1) according to a modified European standard ENV 807 (2009) carried out in two tests namely in vermiculite and soil. The former test is described in Annex A of the standard. Kolle flasks were filled with 125 mL of vermiculite (VWR) having a water holding capacity (WHC) of 322 mL/L. Four pre-weighed wood blocks (i.e. after impregnation and to dryness at 103 °C) were inserted in the vermiculite of each flask and 125 mL of vermiculite added to completely cover the samples. Deionised water was used to ensure 95% of the WHC of vermiculite (i.e. 76.5 mL per flask). Nutrient solution (61.5 mL per flask, see ENV 807 (2009)) was added and the flasks autoclaved for 20 min. The nutrient solution contained 3.00 g NH_4NO_3, 2.56 g K_2HPO_4, 1.02 g $MgSO_4$ x $7H_2O$, 0.25 g KCl, 0.005 g NaCl, 0.001 g $FeSO_4$, 0.001 g $MnSO_4$, 0.001 g $ZnSO_4$, in 1 L water pH 6.2).

The test fungi (Table 1) were grown on MEA plates. One plate of each fungus was homogenized with 100 mL deionised water and 15 mL of the solution inoculated in each Kolli flask. The flasks were weighed weekly to control moisture evaporation during the experiment and deionised water added to ensure the initial weight.

Decay resistance of the treated and untreated samples was additionally tested by a modified ENV 807 (2009) test in soil-jars against the studied soft rot fungi. Glass jars (500 mL) were filled to one-third of their volume with garden soil and four treated and one untreated control sample placed in each jar. After sterilisation,

Table 1
Fungal species, strains and their origin used for decay tests.

Species/Strain	Species origin	Cu-tolerance on agar	Cu-tolerance classification
Phialophora malorum 211-C-15-1	2% K33 impregnated poles, Sweden	10% w/v $CuSO_4$[a]	High Cu-tolerance
Phialophora mutabilis 24-E-1-1	CCA treated transmission poles, Sweden	5.0% w/v $CuSO_4$[a]	Medium Cu-tolerance
Chaetomium globosm F171-1	ATCC 34152	0.0 < 0.1w/v $CuSO_4$[a]	Poor Cu-tolerance

[a] Daniel and Nilsson, 1988.