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Influence of acidification of CCB (Cu/Cr/B) impregnated wood on fungal copper tolerance

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Abstract

Copper tolerant fungi are known for more than 60 years but the complete mechanisms of copper tolerance by these fungi are still not fully understood. Copper tolerance has previously been linked to oxalic acid excretion by copper tolerant brown rot fungi. The oxalic acid then reacts with copper in the wood to form an insoluble and therefore less toxic copper oxalate. It has been suggested that copper tolerance could be due to lowering of the pH of the medium rather than the low solubility of copper oxalate. In order to elucidate this presumption, copper/chromium/boron (CCB) treated wood specimens were acidified with organic (oxalic, acetic, lactic, formic) and inorganic (sulphuric) acids and exposed to copper tolerant (*Antrodia vaillantii*, *Leucogyrophana pinastri*) and copper sensitive (*Poria monticola*, *Gloeophyllum trabeum*) brown rot fungal strains according to the mini block procedure. After eight weeks of exposure, the wood specimens were isolated and their mass losses determined. Additionally, electron paramagnetic resonance (EPR) measurements on the exposed specimens were performed. The EPR spectra of the specimens decayed by *A. vaillantii* were very similar to the EPR spectra of the specimens acidified with oxalic acid. Additionally, acidification of the CCB impregnated specimens made them significantly more susceptible to decay by both the copper tolerant and copper sensitive brown rot fungi.

Keywords: Copper toxicity; Copper tolerance; Wood; Brown rot fungi; Oxalic acid; Wood preservatives

1. Introduction

Copper based solutions have been widely and successfully used for wood preservation for more than a century (Richardson, 1997; Humar et al., 2003). One of the most important issues regarding copper treated wood is the occurrence of copper tolerant fungi (Humar et al., 2002a; Clausen and Green, 2003). In addition to the scientific interest in copper tolerant fungi, there is also a commercial interest in the development of new wood preservatives that would protect wood against these organisms. A further motive for understanding copper tolerance is the possibility of using copper tolerant fungal stains for bioremediation of copper containing waste wood (Stephan et al., 1996; Humar et al., 2004). Despite these interests, the mechanism of copper tolerance by these fungi is still not completely understood.

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Copper tolerance is defined as the ability of an organism to survive copper toxicity by means of intrinsic properties and/or environmental modification of the toxicity (Gadd, 1993). This phenomenon has been linked to oxalic acid production in a number of fungal species (Murphy and Levy, 1983; Sutter et al., 1983). Oxalic acid is involved in fungal-mediated wood-rotting processes (lignocellulose degradation). In preserved wood, it reacts with copper in the wood to form copper oxalate, which is precipitated (Pohleven et al., 1999; Woodward and De Groot, 1999; Humar et al., 2002a). This mechanism is considered to contribute to the detoxification of copper in copper-treated wood and this enables fungi to tolerate environments containing high concentrations of copper and other toxic metals. Since copper oxalate is insoluble, copper in this form has less inhibitory effect on fungal growth (Richardson, 1997; Humar et al., 2002a; Jarosz-Wilkolazka and Gadd, 2003).

On the other hand, it was also suggested that copper tolerance by these fungi had more to do with lowering of the pH of the substrate than with the low solubility of copper oxalate (Young, 1961; Starkey, 1973; Clausen et al., 2000; Humar et al., 2001; Clausen and Green, 2003; Green and Clausen, 2003). It was also demonstrated that *Wolfiporia cocos* exhibited significantly increased tolerance to copper in a nutrient medium when the pH was decreased from 6 to 2 (Young, 1961). However, this experiment was performed on nutrient medium containing all the nutrients required for growth by the fungi. On the other hand, in wood the fungus has to invest a lot of energy to produce all the required nutrients for growth and to colonize the wood. Thus, we were interested in finding out whether acidification of preserved wood has any influence on the ability of fungi to decay it.

2. Materials and methods

Norway spruce (*Picea abies*) samples of dimensions ($0.5 \times 1.0 \times 3.0$ cm) were vacuum impregnated with the 5% CCB solution (34% $CuSO_4 \times 5H_2O$; 37.3% $K_2Cr_2O_7$; 28.7% H_3BO_3) according to the EN 113 procedure (ECS, 1989). The treatment of the wood specimens resulted in a solution uptake of about 400 kg/m^3. Later, the specimens were conditioned for four weeks, the first two weeks in closed chambers, the third week in half closed and the fourth week in open ones. The conditioned specimens were then oven dried (75 °C) for five days in order to ensure complete reduction of the chromium. After conditioning, the specimens were leached according to EN 84 procedure for 14 days (ECS, 1994) and then stored at 25 °C, 65% RH.

The specimens were then acidified by immersing them in jars containing 1%, 5% or 10% aqueous solution of oxalic, sulfuric, lactic, formic or acetic acid (Table 3). After 20 min of vacuum treatment, the specimens were left in the jars for additional two hours. After the acid treatment, the specimens were air dried for one week, oven dried (103 °C) and their masses determined. Finally they were conditioned at 25 °C, 65% RH and steam sterilised prior to exposure to the fungi.

The specimens were exposed to four different brown rot fungi listed in Table 1 according to the non-standard mini block procedure (Pohleven et al., 2000). The fungal cultures were grown and maintained on a 3.9% potato dextrose agar medium (PDA, Difco). Petri dishes (plastic, 9 cm diameter) with PDA medium were inoculated with small pieces of fungal mycelium. When the mycelia overgrew the nutrient medium, three samples of treated and one of control-untreated wood were introduced onto each inoculated petri dish above a plastic net. The specimens were then exposed to fungal decay for eight weeks (Temp. = 25 °C, RH = 75%) after which the fungal mycelia were removed and mass losses of the specimens determined gravimetrically. The specimens were then stored for EPR measurements in a dark and dry place.

EPR measurements were made at room temperature on Bruker ESP-300 X-band spectrometer (microwave frequency = 9.62 GHz, microwave power = 20 mW, modulation frequency = 100 kHz, modulation amplitude = 0.1 mT). Two matchstick like samples ($30 \times 1 \times 1$ mm) were cut from each wood specimen exposed to the fungi as well as from the unexposed ones and inserted one at a time into a resonator. In the case of Cu(II), free radicals and Cr(III) EPR signals, the magnetic parameters of the spin-Hamiltonian equation can be obtained directly from the spectrum. The $g_\perp$ value was obtained from the ratio of the microwave frequency to the value of the magnetic

Table 1
Brown rot fungi used. Copper tolerance was estimated by Pohleven and co-workers (2002a,b)

Fungi	Origin	Estimated Cu tolerance
Antrodia vaillantii	University of Ljubljana, ZIM L037[a]	Highest
Leucogyrophana pinastri	Buckinghamshire Chilterns University College, UK	High
Poria monticola	BAM 102	Low
Gloeophyllum trabeum	University of Ljubljana, ZIM L017[a]	Lowest

[a] Raspor et al. (1995).

field at the zero crossing of the spectrum, and $g_{\|}$ was obtained from the ratio of the microwave frequency to the magnetic field at the centre of the four-line parallel hyperfine pattern. The value of $A_{\|}$, in energy units, is given by the spacing between the components of the hyperfine pattern, measured in mT. However, the value of $A_{\perp}$ is frequently unresolved.

3. Results and discussion

Mass losses caused by the copper sensitive fungi (*G. trabeum* and *P. monticola*) were found to be higher than those caused by the copper tolerant ones (*A. vaillantii* and *L. pinastri*). These results are consistent with those reported by Humar et al. (2004). The reason for the observed differences in mass losses could be due to the different decay mechanism used by the different fungi. However, the copper/chromium/boron (CCB) treatment successfully protected the wood against the copper sensitive strains *G. trabeum* and *P. monticola*. In both cases, mass losses obtained were lower than 2%. On the other hand, exposure of the CCB treated specimens to the copper tolerant fungal strains resulted in significantly higher mass losses between 6.8% and 8.5% (Table 2).

In order to elucidate changes of the active ingredients in the CCB treated wood, EPR analysis of the unexposed and exposed specimens were performed. This method can provide very useful information about changes of paramagnetic centres of Cu(II), Cr(III) and free radicals in wood after certain treatment or fungal decay (Hughes et al., 1994; Pohleven et al., 1994; Humar et al., 2002b). EPR spectra of the unexposed CCB treated wood samples are presented in Fig. 1(a). From this spectra, two different EPR signals can be distinguished: Cr(III) EPR signal ($g = 1.97$, line width = 54 mT) and Cu(II) EPR signal ($g_{\perp} = 2.077$, $g_{\|} = 2.322$, $A_{\perp} = 13.4$ mT). Similar parameters (signals) for CCB treated wood have been previously reported (Hughes et al., 1994; Humar et al., 2002b).

EPR spectra and parameters of the CCB treated specimens decayed by the copper tolerant strain *A. vaillantii* are very similar to the EPR spectra of the specimens that were acidified with aqueous solution of 10% oxalic acid (Table 3, Figs. 1 and 2). After exposure of the treated wood to *A. vaillantii* or acidification, new EPR signals, assigned to free radicals also appeared. Furthermore, it can be observed from both spectra that the copper(II) in the treated wood was completely transformed into copper oxalate ($g = 2.17$) and the broad EPR signal of Cu oxalate can be clearly seen in Figs. 1(c) and (d) and 2. From these spectra, it can be also be seen that all the chromium(III) in the CCB treated wood was completely transformed into chromium oxalate. However, the EPR signal of Cr oxalate can not be resolved from the EPR spectra. Chromium in this form and concentration gives a signal that is too broad to be resolved (Lahiry and Kakkar, 1982; Humar et al.,

Table 2
Mass losses of control and CCB treated specimens acidified with different organic and inorganic acids after eight weeks of exposure to the brown rot fungi

Treatment used	Acid concentration (%)	*A. vaillantii*	*L. pinastri*	*P. monticola*	*G. trabeum*
		Mass loss (%)			
CCB—no acid	0	6.8 (2.2)	8.5 (1.7)	1.6 (1.0)	0.2 (0.2)
CCB oxalic acid	1	10.1 (2.2)	6.3 (1.4)	6.0 (2.6)	5.6 (1.1)
	5	11.1 (1.3)	11.2 (0.4)	11.7 (0.7)	11.0 (1.6)
	10	14.6 (0.7)	14.7 (1.0)	15.5 (0.8)	14.5 (1.1)
CCB acetic acid	1	12.8 (2.1)	13.5 (2.9)	5.2 (0.6)	1.5 (0.3)
	5	13.8 (5.2)	13.5 (5.5)	14.7 (6.1)	6.1 (3.8)
	10	12.0 (2.3)	12.9 (1.5)	7.4 (2.1)	3.2 (0.3)
CCB formic acid	1	11.5 (5.6)	19.7 (4.2)	12.5 (5.3)	4.1 (3.9)
	5	12.5 (0.6)	10.8 (2.5)	4.2 (1.3)	2.1 (0.2)
	10	11.2 (1.1)	15.3 (0.8)	4.8 (1.3)	1.8 (0.1)
CCB lactic acid	1	10.3 (0.5)	12.3 (2.1)	4.7 (2.0)	1.0 (0.1)
	5	14.1 (2.9)	8.1 (2.0)	6.0 (0.6)	4.1 (0.5)
	10	13.6 (4.8)	11.4 (4.8)	11.1 (5.6)	9.2 (5.9)
CCB sulfuric acid	1	7.8 (0.5)	7.3 (0.7)	9.8 (1.5)	6.4 (0.5)
	5	14.1 (1.1)	15.0 (2.0)	15.5 (1.4)	12.9 (1.3)
	10	19.4 (1.7)	19.1 (1.6)	16.0 (3.5)	25.7 (4.9)
Unimpregnated wood—no acid	0	18.5 (5.4)	9.2 (2.3)	40.1 (6.4)	37.1 (7.0)

Standard deviations are given in the parenthesis.

Table 3
Cu(II), Cr(III) and free radical EPR parameters of CCB treated wood after acidification or after exposure to the wood decay fungi

Treatment	Concentration (%)	Cu(II)			Cr(III)		Free radical
		$g_{\|}$	$g_{\perp}$	$A_{\perp}$ (mT)	g	Line width (mT)	Relative intensity
Water		2.077	2.320	13.4	1.97	54	–
Oxalic acid	1	2.076*	–	–	2.00	52	0.49
	5	2.150*	–	–	–	–	1.00
	10	2.170*	–	–	–	–	1.46
Acetic acid	1	2.078	2.322	13.2	1.98	49	–
	5	2.077	2.322	13.2	1.98	37	–
	10	2.066*	2.322	13.2	1.98	36	–
Lactic acid	1	2.080*	2.320	13.5	1.99	55	–
	5	2.083*	2.310	13.5	–	–	0.22
	10	2.086*	2.310	13.5	–	–	0.15
Formic acid	1	2.078	2.320	13.3	1.98	45	–
	5	2.078	2.320	13.3	1.98	39	–
	10	2.078	2.320	13.3	1.99	35	–
Sulfuric acid	1	2.089*	2.372	12.3	2.00	50	4.67
	5	2.089*	2.372	12.0	–	–	20.80
	10	2.089*	2.372	12.0	–	–	31.05
Fungal strain used							
A. vaillantii		2.175*	–	–	–	–	1.52
L. pinastri		2.150*	–	–	–	–	0.98
P. monticola		2.120*	–	–	–	–	0.47
G. trabeum		2.078	–	–	1.99	57	–

– Sign is present when respective signal is not observed at the spectra.
* Sign is present when significant difference from the EPR parameters of CCB treated wood can be denoted.

2004). Similar but less intense changes were also observed for CCB impregnated samples exposed to the copper sensitive strain *P. monticola*. It is also interesting to note, that even though this fungal strain transformed most of the copper in the wood to copper oxalate, it was not able to decay the wood (Fig. 1(c), Table 2). On the other hand, comparing the changes in EPR parameters of the decayed wood with the acidified wood, it can be seen, that changes of the EPR signals caused by the most copper tolerant strain *A. vaillantii* are similar to those of the specimens acidified with the highest concentration of oxalic acid solution. Additionally, changes observed for the specimens exposed to *P. monticola* are similar to those of the specimens acidified with 1% aqueous solution of oxalic acid. These changes are also true for EPR signals of the free radicals (Figs. 1 and 2, Table 3). However, no transformation of the heavy metals into their corresponding oxalates and no free radicals were observed for the samples exposed to *G. trabeum*. (Table 2, Fig. 1). This result was expected since this fungus is copper sensitive and produces very little oxalic acid (Green and Clausen, 2003).

However, changes of the EPR spectra caused by acidification of the specimens with aqueous solution of oxalic acid were significantly different from those of the specimens acidified with acetic, formic, lactic or sulphuric acids of the same concentrations (Fig. 3, Table 3). From the shape and parameters of the EPR spectra of the acidified CCB treated specimens it can be deduced that while some of the acids (lactic and sulphuric) formed new complexes with copper, other acids (formic and acetic) did not. However, these changes were not comparable with those observed with the EPR spectra of the CCB impregnated specimens exposed to the copper tolerant fungal strains.

The main purpose of this research was to investigate how acidification of CCB impregnated wood influences their susceptibility to decay by brown rot fungi. Mass losses of the acidified specimens exposed to the fungi are shown in Table 2. It is clear from the table that acidification of the impregnated specimens increased their susceptibility to decay by both the copper sensitive and the copper tolerant brown rot fungal species. Even acidification of the specimens with the lowest (1%) aqueous solution of oxalic acid resulted in a significantly high mass losses compared to the non-acidified specimens. For example, the copper sensitive *G. trabeum* was not able to decay the CCB impregnated specimens, but after acidification with 1% solution of oxalic acid, a mass loss of 5.6% was obtained. Increasing the concentration of

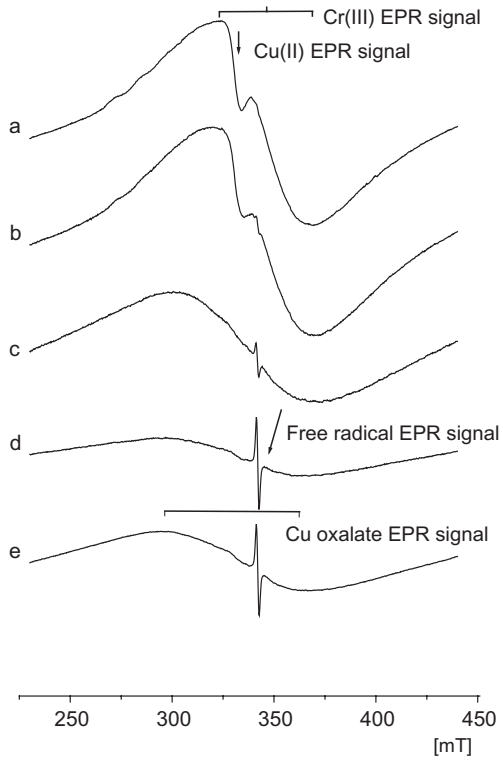

Fig. 1. EPR spectra of CCB treated wood exposed to (a) *G. trabeum*, (b) *P. monticola*, (c) *A. vaillantii*, (d) and *L. pinastri* (e) for eight weeks.

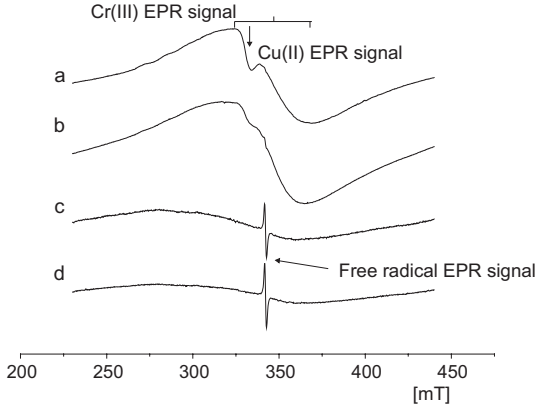

Fig. 2. EPR spectra of CCB treated wood acidified with (a) 1%, (b), 5% (c) and 10% (d) aqueous solution of oxalic acid.

the aqueous solution of oxalic acid increased the mass losses of the specimens. Acidification with oxalic acid also caused an increase in mass losses of the specimens exposed to the copper tolerant strains as well. A mass loss of 14.6% was obtained for the CCB treated specimens acidified with 10% solution of oxalic acid exposed

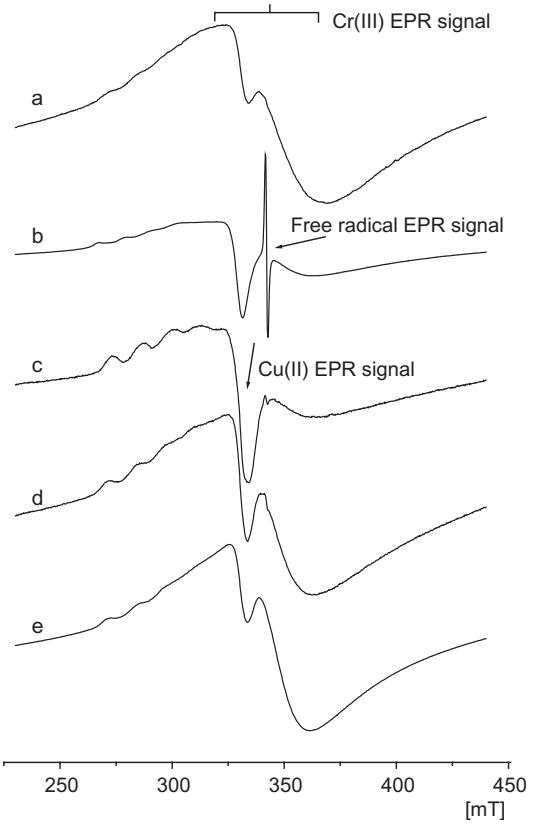

Fig. 3. EPR spectra of CCB treated wood acidified with (a) 5%, aqueous solution of sulfuric (b), lactic (c), formic (d) and acetic acid (e).

to the copper tolerant *A. vaillantii*. This was two times the mass loss obtained for the non-acidified specimens (6.8%). These results show that copper in the form of copper oxalate in wood is less inhibitory to fungal decay compared to copper in the original fungitoxic form (Table 2).

It is very interesting to compare the susceptibility to decay of the impregnated specimens acidified with oxalic acid that reacts with copper in the wood with specimens acidified with the other acids that did not form new complexes with copper in the wood. The EPR spectra of the CCB impregnated specimens acidified with 1% or 5% aqueous solution of formic or acetic acid showed similar shape and parameters of the Cu(II) EPR signal ($g_{\perp}$ = 2.077–2.078, $g_{\parallel}$ = 2.320–2.322, $A_{\perp}$ = 13.0–13.2 mT) to the un-acidified specimens ($g_{\perp}$ = 2.077, $g_{\parallel}$ = 2.322, $A_{\perp}$ = 13.4 mT) (Table 3). This indicates that after acidification with 1% or 5% aqueous solution of acetic or formic acid, the copper was not transformed and it was the acidic environment that influenced the fungal growth. Mass losses of the impregnated specimens acidified with 1% or 5% aqueous solution of acetic or formic acid that

were exposed to the wood decay fungi were notably higher than those of the non-acidified specimens. Even the copper sensitive fungi *G. trabeum* caused a mass loss of 6.1% to the CCB impregnated specimen acidified with 5% solution of acetic acid. This result is consistent with those of Young (1961), Clausen et al. (2000) and Humar et al. (2001) that copper tolerance of these fungi could be due to the lowering of the pH of the medium rather than the low solubility of copper oxalate.

Fungal exposure of the specimens acidified with sulphuric and lactic acids that formed new complexes with copper also resulted in increased mass losses. The highest average mass losses were obtained for specimens acidified with 10% aqueous solution of sulphuric acid. For these specimens, we presume that de-polymerisation of the wood substrate also contributed to the high susceptibility to fungal decay as the wood components were available to the fungi as nutrient sources.

4. Conclusions

EPR spectra of CCB impregnated wood specimens exposed to brown rot fungi *Antrodia vaillantii*, *Leucogyrophana pinastri* and *Poria monticola* are comparable to the EPR spectra of CCB treated specimens acidified with oxalic acid. From these spectra, it is evident that exposure of the CCB treated samples to fungi or acidification of the samples with oxalic acid resulted in the transformation of the bound copper and chromium in the wood to copper oxalate and chromium oxalate respectively.

Acidification of the CCB impregnated specimens with organic (oxalic, acetic, lactic, formic) or inorganic (sulfuric) acid, made them significantly more susceptible to decay by the copper tolerant and copper sensitive brown rot fungi, even when the copper and chromium remained in their original states. We have therefore confirmed that copper tolerance by these brown rot fungi is much more likely due to lowering of the pH of the substrate rather than the low solubility of copper oxalate.

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