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Influence of boron in CCB formulation on growth and decay capabilities of copper tolerant fungi

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Abstract The influence of boron in CCB preservative on infestation of leached and non-leached CCB treated wood specimens by copper tolerant and copper sensitive brown rot decay fungi are described. The tests were performed according the EN 113 procedure using seven different brown rot fungal strains: (*Antrodia vaillantii* (four different strains)*, Poria monticola, Gloeophyllum trabeum* and *Leucogyrophana pinastri*). Leaching of boron from the CCB-treated wood samples rendered them susceptible to decay by the copper tolerant strains but not the copper sensitive ones. Additionally, using EPR spectroscopy, non-toxic copper oxalate was found in samples exposed to the copper tolerant strains (*A. vaillantii* and *L. pinastri*), as well as the copper sensitive strain *P. monticola* but not in the samples exposed to the other copper sensitive strain *G. trabeum.* It is supposed that, in the case of samples that were not leached before exposure to the copper tolerant fungi fungal growth and decay were inhibited by boron.

Einfluss des Bors in CCB-Schutzmitteln auf Wachstum und Abbaukapazität von Kupfer-tolerierenden Pilzen

Zusammenfassung Der Einfluss des Bors in CCB-Schutzmitteln auf den Befall von ausgelaugten und nicht-ausgelaugten, mit CCB behandelten Holzproben durch Kupfer-tolerierende und Kupfer-sensitive Braunfäulepilze wird beschrieben. Die Prüfung erfolgte nach EN 113 mit sieben unterschiedlichen Pilzstämmen: (*Antrodia vaillantii* (vier unterschiedliche Stämme)*, Poria monticola, Gloeophyllum trabeum* und *Leucogyrophana pinastri*). Auslaugen des Bors aus den mit CCB behandelten Proben machte diese anfällig für den Abbau durch Kupfer-tolerante Stämme, nicht jedoch durch Kupfer-sensitive. Mit Hilfe der EPR-Spektroskopie wurde außerdem nicht-toxisches Kupferoxalat in den Proben gefunden, die Kupfer-toleranten Pilzen ausgesetzt waren (*A. vaillantii* und *L. pinastri*) und auch einem Kupfer-sensitiven (*P. monticola*), nicht jedoch dem anderen Kupfer-sensitiven *G. trabeum*. Es ist daher anzunehmen, dass die Pilzhemmung bei Proben, die ohne Auslaugung des Bors den Kupfer-toleranten Stämmen ausgesetzt werden, durch Bor bewirkt wird.

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1 Introduction

Among the brown rot wood decay fungi, copper tolerance is exhibited mostly by strains from the genus *Antrodia.* This phenomenon has been well known and investigated during the past decades (Sharp 1975, Tsunoda et al. 1997, Humar et al. 2001 and 2002). Fungal tolerance of copper was found to be closely related to oxalic acid excretion by the fungi. Oxalic acid, produced by fungal strains, reacts with copper in wood to form an insoluble copper oxalate (Stephan et al. 1996, Humar et al. 2002). Richardson (1997) stated that copper has to be soluble to have a fungicidal effect. However, copper oxalate is insoluble and therefore has no fungicidal effect.

Most of the copper tolerant strains used for this investigation were isolated from copper preserved wood in use. During extensive laboratory testing on nutrient medium containing copper, most of them proved to be copper tolerant (Pohleven et al. 2001 and 2002). However, during further laboratory testing of the strains on CCB impregnated Norway spruce samples, they did not exhibit tolerance (Pohleven et al. 2002). We presume that the reason for this observation could be the presence of boron in the wood. Boron has been shown to leach out from CCB impregnated wood in use (Peylo and Willeitner 1996). The main aim of this work was to elucidate the influence of boron leaching from CCB impregnated wood samples on the wood decay by a number of brown rot fungi.

2 Material and methods

Norway spruce (*Picea abies*) samples of dimensions (1.5×2.5×5 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 samples resulted in a solution uptake of about 360 kg/m^3. Later, the samples 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 samples were then oven dried (75°C) for five days in order to ensure complete reduction of chromium. Following conditioning, the samples were leached according to the EN 84 procedure for 14 days (ECS 1994). Afterwards, the samples were oven dried (103°C), after which their masses were determined and then conditioned at 25°C, 65% RH. The samples were finally steam-sterilized prior to exposure to the fungi.

For this experiment, seven different brown rot fungal strains were used (Table 1). Strains of *Antrodia vaillantii* and *Poria sp.* have been kindly provided by Prof. Dr. Olaf Schmidt (University of Hamburg) and Dr. Ina Stephan (BAM). These fungi had been isolated at various locations of the world. For comparison, copper sensitive strains of *Gloeophyllum trabeum* and *Poria monticola* were also tested. Before the test was performed, the fungi were grown on a 3.9% potato dextrose agar. Jars with PDA medium were inoculated with small pieces of fungal mycelium after which the wood samples (one each of treated and untreated) were placed on a plastic net in each inoculated jar. The samples were then exposed to fungal decay in the growth chamber at 25°C, RH 75% for 16 weeks, after which mycelia were carefully removed from the samples and mass losses determined. The experiment was replicated five times.

EPR experiments were performed at room temperature with Bruker ESP-300 X-band spectrometer (Microwave Frequency = 9.62 GHz, Microwave Power = 20 mW, Modulation Frequency = 100 kHz, Modulation Amplitude = 0.1 mT). Four matchstick like samples (40×1×1 mm) were cut from the wood samples exposed to fungi as well as un-exposed ones. These were inserted one at a time into the resonator.

3 Results and discussion

The mass losses of the control un-impregnated samples (Table 2) show that all fungi used for the test were virulent. Mass losses caused by the copper sensitive fungi (Gt2 and Pm2) were found to be higher than those caused by the copper tolerant ones. This could probably be due the type of decay mechanism used by the fungi. Brown rot fungi deteriorate wood substrate by non-enzymatic and enzymatic mechanisms. The non-enzymatic type of decay is due to free radical generation via Fenton reaction. This type of decay is particularly important during the initial

Table 1 Isolates of fungi tested for copper tolerance
Tabelle 1 Prüfung der einzelnen Pilzstämme aus Kupfer-Toleranz

Fungi	Abbreviation	Origin	Cu tolerance[a]
Antrodia vaillantii	Av 4	University of Hamburg DFPG 6911 New Zealand	Cu tolerant
Antrodia vaillantii	Av 5	University of Hamburg HUM Germany	Cu tolerant
Antrodia vaillantii	Pv 2	University of Ljubljana ZIM L037 (Raspor et al. 1995)	Cu tolerant
Antrodia vaillantii	Pv 4	BAM 190	Cu tolerant
Poria monticola	Pm2	BAM 102	Cu sensitive
Leucogyrophana pinastri	Yf	Buckinghamshire Chilterns University College UK	Cu tolerant
Gloeophyllum trabeum	Gt 2	University of Ljubljana ZIM L017 (Raspor et al. 1995)	Cu sensitive

[a] Pohleven et al. 2001 and 2002

Table 2 Average mass losses of Norway spruce wood samples: impregnated with CCB solution (CCB) and afterwards leached (CCB leached) and unimpregnated ones (control) exposed to different brown rot fungi for 16 weeks. Standard deviations are given in the parentheses

Tabelle 2 Durchschnittlicher Massenverlust von Fichtenproben nach 16-wöchigem Befall mit verschiedenen Braunfäulepilzen: imprägniert mit CCB und danach ausgelaugt sowie nicht-imprägnierte Kontrollproben. (Standardabweichungen sind in Klammern angegeben)

Treatment	Fungal strain						
	Pv2	Pv4	Av5	Yf	Av4	Pm2	Gt
	Mass loss [%]						
CCB	1.8 (0.3)	1.4 (0.2)	1.1 (0.2)	1.2 (0.3)	1.2 (0.2)	0.6 (0.2)	1.4 (0.3)
CCB Leached	4.3 (0.9)	8.9 (4.4)	4.8 (1.7)	2.4 (2.2)	6.3 (2.1)	0.0 (0.1)	0.1 (0.0)
Control	31.0 (1.8)	22.4 (3.3)	25.0 (3.5)	20.3 (2.2)	24.9 (3.0)	47.2 (5.4)	43.3 (2.5)

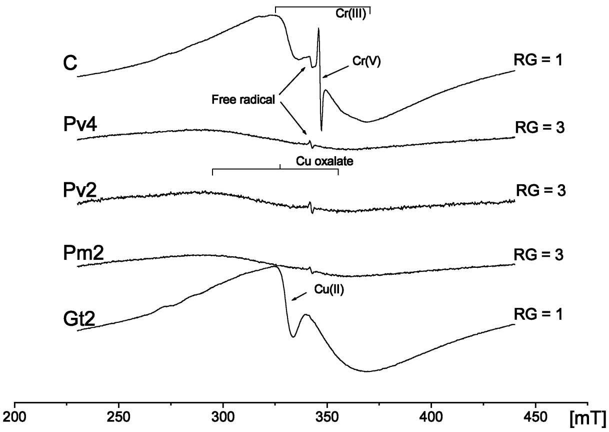

Fig. 1 EPR spectra of CCB treated Norway spruce specimens: unexposed (*c*) and exposed to the copper tolerant fungi *A. vaillantii* (Pv4 and Pv2) and to copper sensitive *P. monticola* (Pm2) and *G. trabeum* (Gt2) for 16 weeks

Abb. 1 EPR-Spektren der mit CCB behandelten Fichtenproben nach 16-wöchigem Befall mit Kupfer-toleranten Pilzen (*A. vaillantii* (Pv4 and Pv2) sowie Kupfer-sensitiven Pilzen *P. monticola* (Pm2) und *G. trabeum* (Gt2): Kontrollproben (*c*)

stages of decay when the pores in the wood cell wall matrix are too small for enzymes to penetrate. However, the Fenton type reaction is very sensitive to pH, thus high oxalic acid excretion by the copper tolerant fungi will disable it, and would result in low mass losses (Schmidt et al. 1981, Green III et al. 1991).

None of the fungal strains investigated was able to decay the samples impregnated with CCB. Mass losses obtained for samples infested by both the copper sensitive and copper tolerant strains were less than 2% (Table 2). On the other hand, exposure of leached CCB treated samples to the fungi resulted in significantly higher mass losses. The highest mass losses were recorded for samples exposed to the copper tolerant *A. vaillantii* (Pv4) (8.9%) with 2.4% and 6.3% recorded for Yf and Av4 respectively. These mass losses are significantly higher than those recorded for samples exposed to non-leached samples (Table 2). We believe, that the reason for the higher mass losses observed for the leached CCB treated samples could be due to the leaching of boron from the wood. Peylo (1995) reported that about 80% of boron could be leached from treated wood after four weeks of leaching. In contrast, Humar with coworkers (2003) reported significantly less leaching of chromium and copper losses (2%) from treated wood. However, mass losses obtained for the samples infested by the copper sensitive *P. monticola* and *G. trabeum* (Table 2) were insignificant This result indicates that the active substances left in the leached CCB treated wood were sufficient to protect the wood against the copper sensitive species, but not the copper tolerant ones.

EPR spectra of the leached and unleached CCB treated specimens infested by copper tolerant strains (Pv2, Pv4, Av4, Av5 and Yf), showed that all the copper (II) in the treated wood was completely transformed into copper oxalate which is insoluble and non-toxic, resulting in fungal growth and wood decay. It can clearly be seen from Fig. 1 that copper oxalate gives a very broad signal. The spectrum also shows that chromium(III) in the CCB treated wood was completely transformed into chromium oxalate. However, chromium in this form and concentration could not be resolved from the spectra, as its signal is too broad (Humar et al. 2002). The same changes were observed for CCB impregnated samples exposed to the copper sensitive strain *P. monticola.* On the other hand, no transformation of the heavy metals into oxalates was observed for samples exposed to the copper sensitive *G. trabeum.* These results indicate that though transformation of copper-to-copper oxalate is essential, it could not be the only mechanism responsible for copper tolerance by these fungi. It is also interesting to note that though the copper sensitive fungus *P. monticola* transformed all the copper in the wood samples to copper oxalate, it could not decay the wood. (Table 2, Fig. 1). It is clear that with the non-leached samples, inactivation of copper by transforming it to copper-oxalate was could not render the wood susceptible to decay. This could be due to the presence of boron in the samples, which inhibited fungal growth and wood decay.

4 Conclusions

Boron in CCB preserved wood have been found to influence the growth of copper tolerant strains after the other heavy metals have been inactivated through the formation of oxalates. Virtually no growth was observed on unleached CCB impregnated samples exposed to the copper tolerant strains. After leaching of the wood, both in laboratory, or/and in service conditions, boron leaches out and the wood becomes susceptible to decay by the copper tolerant fungi but not the copper sensitive strains. Furthermore, the transformation of copper into copper oxalate by the fungi was found to be essential but not the only mechanism responsible for copper tolerance by these fungi.

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