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Changes in EPR Spectra of Wood Impregnated with Copper-Based Preservatives during Exposure to Several Wood-Rotting Fungi

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Summary

The tolerance of various fungi against copper was examined. For this purpose, we impregnated Norway spruce (*Picea abies*) specimens with two different aqueous solutions: copper(II) octanoate with ethanolamine or copper(II) sulfate ($c_{Cu} = 1.0 \times 10^{-2}$ mol/l). Impregnated and unimpregnated test specimens were then exposed to brown rot fungi *Antrodia vaillantii* and *Gloeophyllum trabeum* or to white-rot fungi *Schizophyllum commune* and *Trametes versicolor*. After 2, 4, 6 and 12 weeks of exposure Electron Paramagnetic Resonance, Atomic Absorption Spectroscopy and mass loss measurements were performed. The results indicate that *A. vaillantii*, *G. trabeum* and *T. versicolor* transform copper(II) sulfate in wood into non-soluble, and therefore non-toxic, copper oxalate. The intensity of this reaction depends on the amount of excreted oxalic acid and was the highest for *A. vaillantii* and the lowest for *T. versicolor.* In the presence of ethanolamine, formation of insoluble copper oxalate was not possible and therefore, decay could not proceed. The major portion of copper remained in the wood and only minor amounts were in some cases translocated into nutrient media.

Introduction

Copper-based formulations are among the most important and frequently used commercial wood preservatives and have been widely and successfully applied for more than a century. Although the nature of the toxicity of copper to fungi is still not well understood, it is clear that copper must be dissolved in water to be delivered to the fungal spore or cell. The soluble portion of copper compounds which remain undissolved act as a reservoir from which copper ions are released to the substrate (Sharp 1975; Richardson 1997; Pohleven *et al.* 1999).

Several brown rot fungi reveal tolerance to copper, and consequently copper-based wood preservatives may not be sufficient. The existence of copper-tolerant fungi has been known for quite some time (Hirt 1949; Zabel 1954; Da Costa 1959). Copper tolerance can be defined as the ability of an organism to survive copper toxicity by means of intrinsic properties and/or environmental modification of toxicity. This means that the tolerant fungi can grow on copper-containing substrates where the concentration of copper is higher than 1.6 mmol/l (Gadd 1993). The wood-destroying basidiomycetes that are tolerant to copper or copper-based preservatives include some species of the genus *Antrodia* (*Poria*). The highest copper tolerance was found in some strains of the wood-rotting fungus *A. vaillantii*. (Sutter *et al*. 1983; Collett 1992; Tsunoda *et al*. 1997; Woodward and De Groot 1999). Tolerance was exhibited both in laboratory experiments and in service situations. For this reason, *A. vaillantii* is widely used as a test fungus for biological evaluation of copper-containing wood preservatives. (Sutter *et al*. 1983)

The resistance of some species of wood decay fungi to copper has been connected to their production of copious amounts of oxalic acid (Murphy and Levy 1983). Oxalic acid is a two-basic organic acid with two low pK values ($pK_1 = 1.27$; $pK_2 = 4.26$) (Skoog *et al*. 1992). It is often produced in great quantities by brown rot fungi (Takao 1965; Green *et al*. 1991), and is associated with brown rot colonization of wood (Jellison *et al*. 1997). It is also the by-product of lignin degradation by white-rot fungi (Kirk and Cullen 1998). Illman and Highley (1996) suggested that oxalic acid reacts with copper and forms insoluble copper oxalate.

Copper-tolerant organisms are of great interest from two different perspectives. Firstly, knowing the exact mechanisms of tolerance, would allow development of new, more efficient preservatives. On the other hand, we could use copper-tolerant organisms for the bio-recycling of copper-containing waste wood through bioremediation, biodeterioration and bioconversion. This is especially interesting as the life cycle of treated wood is estimated to be about 25 years: after which wood is discarded as waste (Illman and Highley 1996). By the year 2020, worldwide about 19×10^6 m^3/year of copper treated wood will be available for recycling (Felton and De Groot 1996).

In order to elucidate the mechanisms responsible for copper tolerance of some wood decay fungi, electron paramagnetic resonance (EPR) was used to investigate the transformation of copper complexes in wood during degradation due to the action of fungi. EPR is a useful technique for the investigation of chemical species with unpaired electrons, such as radicals and some transition metal ions. In the field of wood science and technology, EPR has been found a useful method, either for the study of decay processes in untreated wood (Illman *et al.* 1988; Qian and Goodell 2000) or wood preservatives in undecayed wood (Pohleven *et al.* 1994; Hughes *et al.* 1994). However, to our best knowledge there are no reports in the literature on the use of EPR for investigation of changes in wood and copper-based preservatives caused by fungal degradation.

Materials and Methods

Specimens of Norway spruce (*Picea abies* (L). Karst) sapwood, sized 3.0 × 1.0 × 0.5 cm were impregnated with two different copper water-borne preservatives. The concentration of copper was 1.0×10^{-2} mol/l in both solutions. This concentration was chosen as it slowed but did not stop the growth of fungi. Concentrations of copper in commercial fungicides are at least ten times higher. We used one acidic solution [copper(II) sulfate, (CuS) pH = 3.5] and one alkaline [copper(II) octanoate with ethanolamine (CuE) pH = 11.0]. Control specimens were impregnated with distilled water.

Specimens were vacuum impregnated and prepared according to the EN 113 procedure (ECS 1989). Treatment of small blocks resulted in a preservative uptake of about 550 kg/m^3 of the oven dry wood mass. For this investigation the following brown rot fungi were used: *Gloeophyllum trabeum* (Pers. Ex Fr.) Murill (ZIM L017) (Raspor *et al.* 1995) and two isolates of the same species *Antrodia vaillantii* (DC.: Fr) Ryv. (P163 HUM UH (*A. v.* 1) and ZIM L037 (*A. v.* 2)) (Raspor *et al.* 1995). The first strain was kindly provided by Professor Olaf Schmidt from BFH Hamburg. The white rot species were *Trametes versicolor* (L.: Fr.) Pilát (recently isolated strain) and *Schizophyllum commune* (Fr. ex Fr.) (ZIM L042) (Raspor *et al.* 1995). Both strains of *A. vaillantii* are, according to our previous investigations, copper-tolerant (Humar *et al.* 2001a). The cultures were maintained on solid plating media, which contained 3.9 % potato dextrose agar powder (PDA Difco). Petri dishes with PDA medium were inoculated with small pieces of fungal mycelium. After drying, five specimens of treated and untreated wood were put into each petri dish under plastic net and exposed to fungal decay (growth chamber, dark, 25 ± 1 °C, RH = 75 %) for 2, 4, 6 and 12 weeks.

After exposure to fungi, the spruce wood specimens were isolated. Two specimens were used to determine the rate of decay and were subsequently used for atomic absorption spectroscopy (AAS) measurements, and the other three for electron paramagnetic resonance (EPR) measurements. EPR experiments were performed at room temperature on a Bruker ESP-300 X-band spectrometer (Microwave Frequency = 9.62 GHz, Microwave Power = 20 mW, Modulation Frequency = 100 kHz, Modulation Amplitude = 0.1 mT). We cut off all four corners of each wood specimen (3 × 1 × 1 mm) and inserted them and one by one into a resonator. Therefore, EPR measurements of each observation were performed twelve times in parallel. Decay was determined gravimetrically. For AAS measurements, specimens of culture media and wood were dried at 103 ± 2 °C. Afterwards, 0.25–0.5 g of dry specimen was digested with an acid mixture (5 ml HNO_3 conc. and 2 ml H_2O_2) in closed teflon vessels heated in a microwave oven. Digested specimens were transferred to 50 ml volumetric flasks with water. Cu was determined in specimen solutions with FLAAS (Thermo Yarrel Ash Scan 1). Concentrations of copper in nutrient media are expressed in µg of copper per gram of isolated undried material, while concentrations of copper in decayed wood were expressed in µg of copper per gram of oven dry undecayed wood. Therefore, we could not compare concentrations of copper in nutrient media with copper in wood.

Some fungi are able to produce an acid environment (Green *et al.* 1991). Therefore, we additionally treated six impregnated and six unimpregnated specimens with a solution of oxalic acid or anaqueous solution of sulfuric acid (H_2SO_4) (pH = 1.0). Specimens were vacuum treated with acid by the EN 113 procedure (ECS 1989). After treatment, the specimens were placed into closed petri dishes for 16 hours, and afterwards EPR measurements were performed as described above. On the surface of copper(II) sulfate treated specimens additionally treated with oxalic acid a blue powder appeared. FTIR spectra of this powder were measured on mineral oil mulls between Csl plates using Perkin Elmer FT-IR 1720X in the range of 4000 to 400 cm^{-1}.

Results and Discussion

Untreated wood

Mass loss in control samples is presented in Table 1. For most of the fungi, a manganese Mn(II) signal appeared in the EPR spectrum ($g_0 = 2.003$ $a_0 = 9.6$ mT) (Hughes *et al.* 1994) (Fig. 1a) when the first mass loss was traced. This signal was present in most of the spectra of untreated specimens exposed to brown rot or white rot fungi. The same manganese EPR signal was observed also in unimpregnated specimens, which were treated with oxalic acid (Fig. 1c) or sulfuric acid and not exposed to fungi. Therefore, the presence of the manganese EPR signal in wood is strongly correlated to the low pH of the substrate. Acidification of wood may be caused either by exposure to wood-destroying fungi, which excrete oxalic acid, or a mixture of different organic acids (Takao 1965; Humar *et al.*, 2001a) or by treatment of wood with a solution of oxalic acid or any other acid, as for example sulfuric acid. In all cases, an EPR signal of manganese appeared (Fig. 1). The concentration of manganese in decayed specimens is statistically not different from concentrations of manganese in untreated or oxalic treated wood (Petrič *et al.* unpublished). Therefore, we suggest that the translocation of manganese from fungal mycelium or nutrient media is not the reason for the detection of a Mn(II) manganese signal. Together with the manganese EPR signal, another signal, centered at $g_0 = 2.003$ appeared in specimens exposed to the white rot fungi *T. versicolor* (Fig. 1b). This signal is within the range specific for heteroatomic organic free radicals containing oxygen. Such free radicals may originate from fungal degradation of wood or its components (Dobbs 1974; Harańczyk, 1999; Kirk and Cullen, 1998).

Copper-treated wood exposed to Antrodia vaillantii

We did not observe any significant differences between the two strains of A. vaillantii. Therefore, the results for both strains are presented together.

Table 1. Decay and moisture content (MC) of impregnated and unimpregnated wood exposed to wood rotting fungi. Decay is expressed as percentage of mass loss with respect to the initial mass (MC in brackets)

Treatment formulation	Fungus	Decay [%] and MC [%] (MC in brackets)			
		Time of exposure [weeks]			
		2	4	6	12
Aqueous solution of copper(II) sulfate	*T. versicolor*	0.0 ± 0 (26)	0.0 ± 0 (21)	0.0 ± 0 (30)	1.0 ± 0.3 (48)
	S.commune	0.0 ± 0 (37)	0.0 ± 0 (26)	0.0 ± 0 (26)	0.0 ± 0 (54)
	A.vaillantii 1	0.7 ± 0.3 (26)	4.2 ± 0.4 (27)	3.9 ± 0.8 (39)	11.7 ± 1.1 (37)
	A.vaillantii 2	2.1 ± 0.6 (19)	6.7 ± 0.7 (23)	7.7 ± 0.9 (26)	9.0 ± 0.9 (43)
	G. trabeum	0.0 ± 0.1 (39)	3.9 ± 0.3 (36)	12.8 ± 1.8 (47)	31.4 ± 2.3 (60)
Aqueous solution of copper(II) octanoate and ethanolamine	*T. versicolor*	0.0 ± 0 (48)	0.0 ± 0 (44)	0.0 ± 0 (40)	0.0 ± 0 (80)
	S.commune	0.0 ± 0 (57)	0.0 ± 0 (36)	0.0 ± 0 (44)	0.0 ± 0 (102)
	A.vaillantii 1	0.0 ± 0 (57)	0.0 ± 0 (44)	0.0 ± 0 (47)	0.0 ± 0.1 (128)
	A.vaillantii 2	0.0 ± 0 (34)	0.0 ± 0 (40)	0.0 ± 0 (41)	3.2 ± 0.5 (80)
	G. trabeum	0.0 ± 0 (57)	0.0 ± 0 (65)	0.0 ± 0 (86)	0.0 ± 0.1 (61)
water (control)	*T. versicolor*	2.4 ± 0.5 (25)	4.7 ± 0.7 (34)	13.9 ± 1.2 (39)	22.8 ± 2.4 (73)
	S.commune	0.0 ± 0.0 (33)	0.0 ± 0.0 (24)	0.0 ± 0.0 (25)	0.0 ± 0.0 (47)
	A.vaillantii 1	0.9 ± 0.2 (27)	7.4 ± 0.8 (26)	5.9 ± 0.4 (27)	7.9 ± 0.9 (39)
	A.vaillantii 2	2.0 ± 0.5 (17)	6.3 ± 0.7 (28)	7.9 ± 0.9 (25)	14.2 ± 1.3 (39)
	G. trabeum	2.5 ± 0.8 (32)	10.0 ± 0.9 (45)	28.5 ± 3.1 (55)	38.1 ± 4.7 (72)

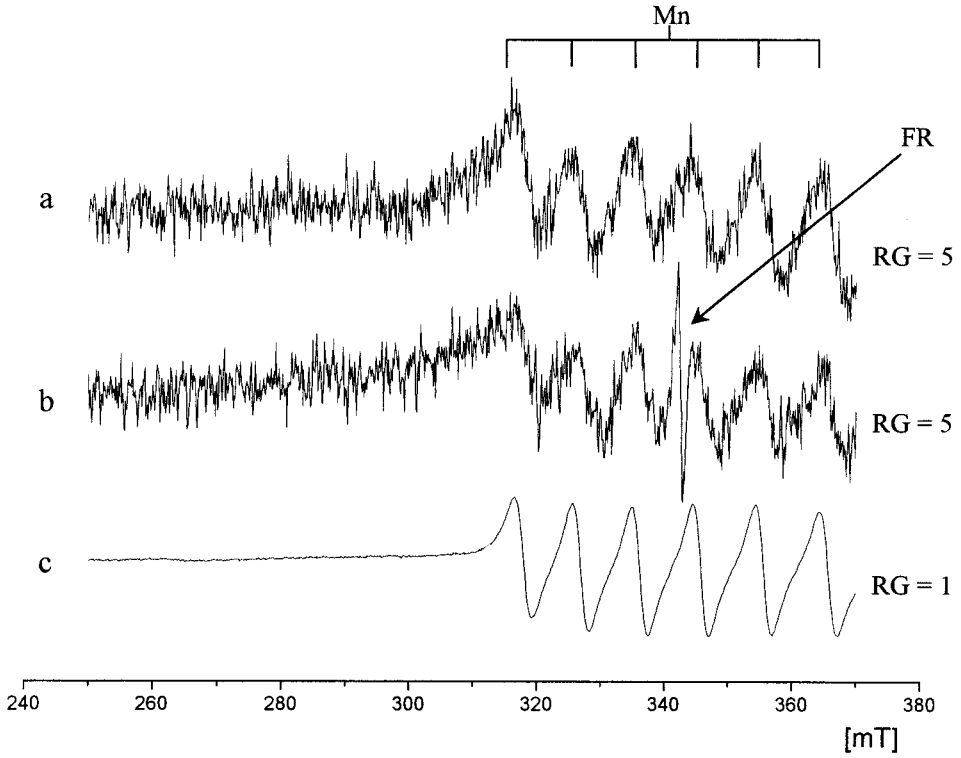

Fig 1. EPR spectrum of unimpregnated sample exposed to *A. vaillantii* for 2 weeks (a), unimpregnated sample exposed to *T. versicolor* for 6 weeks (b) and spectrum of unimpregnated wood treated with a solution of oxalic acid without exposure to a fungus (c). RG is relative receiver gain with respect to spectrum c.

Wood treated with Copper(II) sulfate

Wood impregnated with copper(II) sulfate (CuS) exhibits an anisotropic Cu(II) EPR spectrum ($g_I = 2.079$; $g_{II} = 2.366$; $a_{II} = 12.5$ mT) (Fig. 2a). However, two weeks of exposure to copper-tolerant wood-destroying fungi was enough to produce a pronounced decrease in the EPR signal of copper(II) sulfate in wood (Fig. 2b). At the same

time, mass loss could be traced (Table 1). As decay proceeded, the copper signal completely disappeared. Instead of the copper spectra, a weak signal, typical for Mn(II) appeared (Fig. 2c, d, e). These results show that the fungus *Antrodia vaillantii* transformed copper(II) sulfate in wood into another form that could not be detected by EPR. We think that this form might be copper oxalate. Namely, when we additionally treated copper(II) sulfate impregnated wood with oxalic acid and did not expose it to *A. vaillantii*, the same changes in the EPR spectra occurred: we could no longer detect the Cu(II) signal, but observed a manganese signal instead. At the same time, on surfaces of the specimens a blue powder appeared. EPR spectrum of this powder had the same lineshape as reported in the literature for copper oxalate (Srivastava *et al.* 1980), with a measured g_0 value of 2.175 and linewidth about 43 mT. However, the concentration of Cu(II) in treated wood was too low for such a broad signal to be resolved on the EPR spectra. Nevertheless, a broad Cu(II) oxalate EPR signal is slightly indicated in Figure 2e. Additionally, the FTIR spectrum of surface crystals was recorded. Absorption peaks correspond well with the reported data for copper(II) oxalate (Kuroda and Kubo 1960; Edwards *et al.* 1991). Some other authors have also reported that copper(II) sulfate reacts with oxalic acid to give copper oxalate (*e. g.* White *et al.* 1997). Therefore, in copper(II) sulfate treated wood subsequently treated with oxalic acid copper oxalate is formed. In the same way, the results obtained from copper(II) sulfate impregnated wood exposed to *A. vaillantii* can be explained. Therefore, we conclude that copper in wood reacts with oxalic acid excreted by *Antrodia vaillantii* to give copper oxalate and that copper is not translocated from wood into mycelium and/or nutrient substrate. Copper oxalate is almost insoluble in water (White *et al.* 1997) so it is not, or less, toxic to fungi.

This conclusion is further supported by AAS analysis of copper(II) sulfate treated and exposed wood which showed that copper is present in wood even though we could not observe its EPR signal. There were not any statistically significant differences between concentrations of copper in impregnated specimens exposed to *A. vaillantii* for 12 weeks (629 ppm) and impregnated unexposed specimens (650 ppm) (Table 2). In addition, concentrations of copper in nutrient media after 12 weeks of decay of Cu(II) sulfate impregnated specimens were comparable with concentrations of Cu in nutrient media after 12 weeks of decay of control specimens, 0.6 and 0.5 ppm, respectively. All these results prove that translocation of copper from wood to nutrient media did not occur in this case.

Wood treated with Copper(II) octanoate and ethanolamine

Wood impregnated with the aqueous solution of copper(II) octanoate/ethanolamine (CuE) was less affected than untreated wood or wood treated with copper(II) sulfate. Twelve weeks of exposure to *A. vaillantii* resulted in only 3 % decay (approximately five times less than the mass loss of control specimens) (Table 1). Despite minimal decay, changes in the EPR spectra were more prominent. Figure 3a shows a typical EPR spectrum of wood impregnated with copper(II) octanoate ($g_I = 2.062$; $g_{II} = 2.259$; $a_{II} = 16.5$ mT). After two weeks of exposure, the EPR spectrum changed. Instead of an anisotropic spectrum, we can see a typical isotropic Cu(II) EPR spectrum ($g_0 = 2.151$ $a_0 = 6.2$ mT) (Fig. 3b). This EPR signal has the same parameters as the EPR signal of an aqueous solution of copper(II) octanoate, oxalic acid and ethanolamine, which differs from the isotropic spectrum of aqueous solution of copper(II) octanoate/ethanolamine without oxalic acid ($g_0 = 2.105$ $a_0 = 7.1$ mT). This shows that both copper in wood and copper in preservative solution were dissolved,

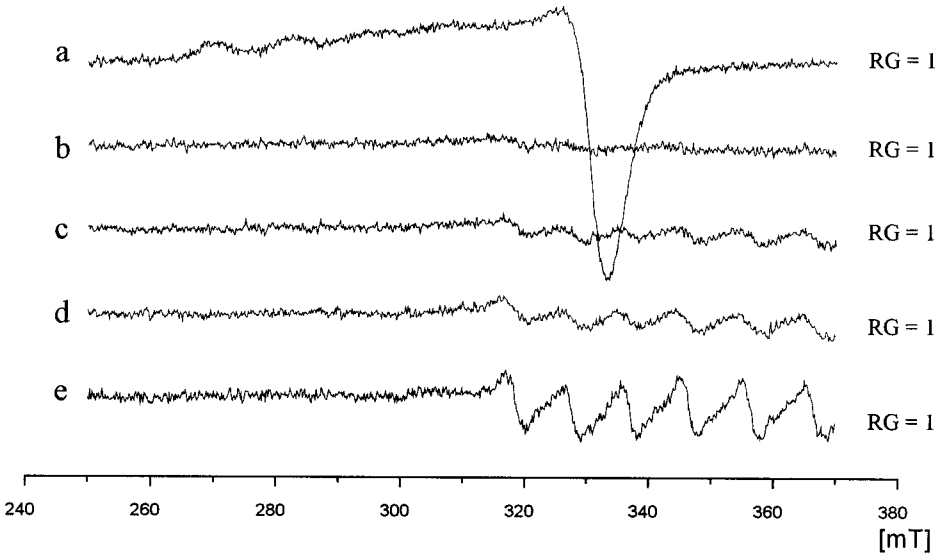

Fig. 2. EPR spectra of wood impregnated with aqueous solution of copper(II) sulfate (a), and exposed to *A. vaillantii* for two (b), 4 (c), 6 (d) and 12 weeks (e). RG is a relative receiver gain with respect to spectrum a.

Table 2. Concentration of copper in impregnated and unimpregnated wood and in solid nutrient media (agar) after 2, 4, 6 and 12 weeks of exposure to *A. vaillantii, G. trabeum* and *T. versicolor*

Treatment formulation	Substrate		*Concentration of Cu [ppm]*				
			Time of exposure [week]				
			0	2	4	6	12
Aqueous solution of copper(II) sulfate	wood	*A. vaillantii*	650 ± 100	635 ± 100	575 ± 100	524 ± 100	670 ± 100
		G. trabeum	650 ± 100	690 ± 100	531 ± 100	527 ± 100	562 ± 100
		T. versicolor	650 ± 100	593 ± 100	639 ± 100	687 ± 100	615 ± 100
	agar	*A. vaillantii*	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.6 ± 0.1
		G. trabeum	0.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
		T. versicolor	0.2 ± 0.1	1.1 ± 0.1	3.6 ± 0.4	17.6 ± 2.1	30.8 ± 2.1
Aqueous solution of copper(II) octanoate and ethanolamine	wood	*A .vaillantii*	632 ± 100	679 ± 0.1	725 ± 100	710 ± 100	594 ± 100
		G. trabeum	632 ± 100	495 ± 100	732 ± 100	718 ± 100	742 ± 100
		T. versicolor	632 ± 100	611 ± 100	593 ± 100	607 ± 100	582 ± 100
	agar	*A. vaillantii*	0.2 ± 0.1	2.5 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	9.7 ± 0.1
		G. trabeum	0.2 ± 0.1	3.5 ± 0.1	3.9 ± 0.4	103 ± 30.4	59 ± 10.4
		T. versicolor	0.2 ± 0.1	10.3 ± 2.1	28.6 ± 2.1	42.1 ± 7.4	50.3 ± 5.4
water (control)	wood	*A. vaillantii*	0.0 ± 1	0.0 ± 1	2.0 ± 1	0.5 ± 1	0.0 ± 1
		G. trabeum	0.0 ± 1	0.0 ± 1	0.0 ± 1	0.0 ± 1	0.0 ± 1
		T. versicolor	0.0 ± 1	0.0 ± 1	0.0 ± 1	1.2 ± 1	0.0 ± 1
	agar	*A. vaillantii*	0.2 ± 0.1	0.6 ± 0.1	0.0 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
		G. trabeum	0.2 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
		T. versicolor	0.2 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1

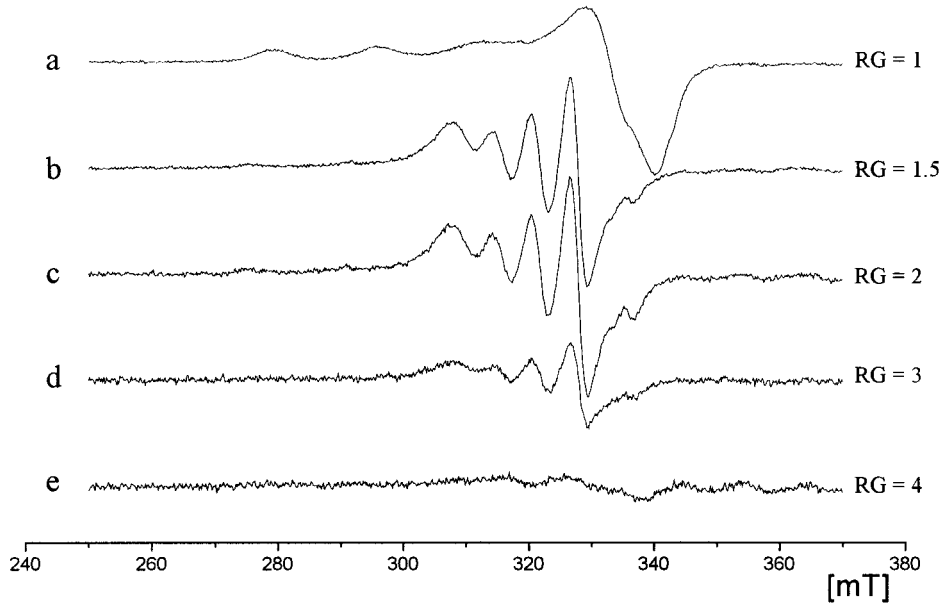

Fig. 3. EPR spectra of wood impregnated with the aqueous solution of copper(II) octanoate/ethanolamine (a), and exposed to *A. vaillantii* for 2 (b), 4 (c), 6 (d) and 12 weeks (e). RG is a relative receiver gain with respect to spectrum a.

but coordination around copper ions in the investigated systems is not the same. The same spectrum, as shown in Figure 3b, was also obtained after four weeks of exposure (Fig. 3c). However, after twelve weeks of exposure, the copper signal disappeared and a weak manganese EPR spectrum was observed instead (Fig. 3e). After twelve weeks of exposure, also the first mass loss was observed (Table 1). If we treated unexposed wood impregnated with CuE with a solution of oxalic acid, we got the same isotropic spectra as for the treated wood exposed to fungi for 2, 4 and 6 weeks. We assume that *A. vaillantii* is not able to transform copper(II) into an insoluble, harmless complex in the

presence of ethanolamine, at least in the first weeks of fungal attack. According to Richardson (1997), copper must be dissolved to have a fungicidal effect.

We did not observe a significant decrease in the copper concentration of the wood specimens. We observed that the percentage of copper in agar increased constantly during the exposure of CuE treated specimens, from an initial 2.5 ppm after 2 weeks of exposure, to a final 9.7 ppm after 12 weeks of rotting, indicating that a minor portion of copper was translocated into the nutrient medium. This final concentration was approximately ten times higher than the concentration of Cu in nutrient media after 12 weeks of control specimen decay (0.5) (Table 2). We presume that a minor part of the copper in wood had to be translocated from the specimens into nutrient medium and the greater portion of the copper was transformed into an insoluble form, possibly copper oxalate, and remained in the specimens. That translocation occurred only in wood impregnated with CuE, but not in samples impregnated with CuS, may be the result of the previously described role of ethanolamine in CuE impregnated wood. In the presence of ethanolamine, fungi (with excretion of oxalic acid) cannot make copper insoluble, and therefore translocation can take place. On the other hand, in a copper(II) sulfate treated wood, copper in the wood reacts with oxalic acid and an insoluble copper oxalate is formed which cannot be transported by fungi out of the wood. We do not yet know what kind of translocation takes place. However, in our opinion there are two possible explanations for this phenomenon. First, the fungi could actively transport copper out of wood. The other alternative is that fungi increase the moisture content of wood and copper is translocated into nutrient media by diffusion.

While the moisture content of unexposed, unimpregnated spruce wood and wood treated with copper(II) sulfate or copper(II) octanoate/ethanolamine was approximately the same (in the range from 11–14 % at 65 % relative air humidity), it is interesting to note that substantial differences in moisture content appeared during decay (Table 1). Generally, the moisture content of copper(II) octanoate/ethanolamine treated wood during decay was approximately two times higher than that of copper(II) sulfate treated wood or control specimens. However, fungi were trying to optimize the pH of wood, changing it from an alkaline to an acidic pH by moisturizing and excreting organic acids. We noticed the same increased moisture content during exposure to *A. vaillantii* in specimens treated only with an alkaline aqueous solution of NaOH (Humar *et al.* unpublished results 2001).

Copper-treated wood exposed to Gloeophyllum trabeum

G. trabeum is an important and very efficient brown rot wood-destroying fungi. However, despite its good rotting ability, this fungus produces three times less oxalic acid than *A. vaillantii*. Instead, this fungus excretes other organic acids (Takao 1965). This was also observed in our EPR spectra of decayed, impregnated wood.

Wood treated with Copper(II) sulfate

Wood impregnated with the aqueous solution of copper(II) sulfate (CuS) was less destroyed than control specimens were. For example, 6 weeks of exposure of CuS treated wood resulted in 2 times lower mass loss (12.8 %) than in control specimens (28.5 %). After 2 weeks of exposure to *G. trabeum,* no mass loss was observed, but the first change was observed in the EPR spectra. The copper EPR signal was overlapped by the manganese spectrum (Fig. 4b). The intensities of manganese spectra increased constantly during the exposure. On

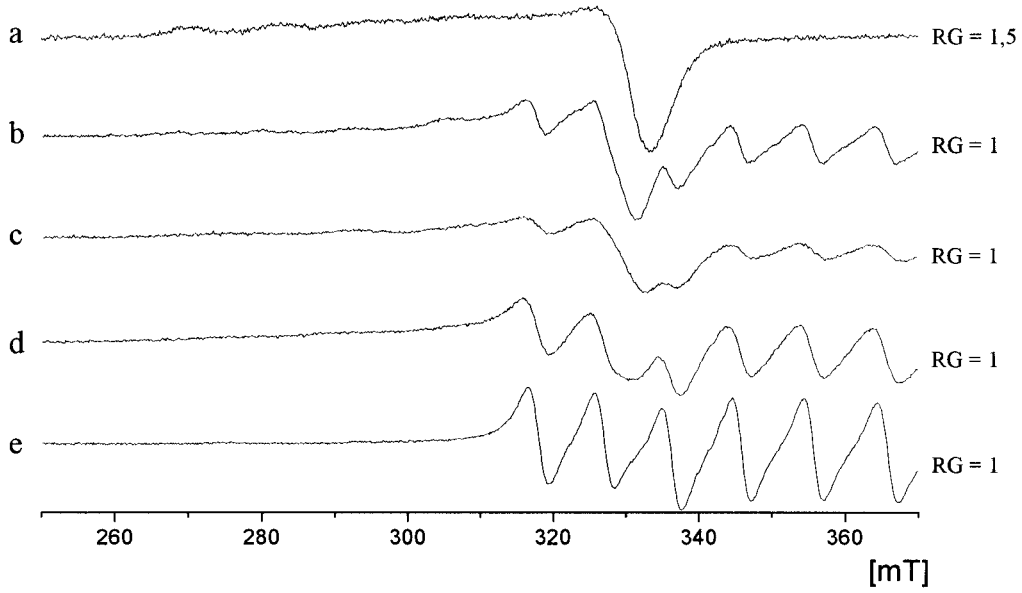

Fig. 4. EPR spectra of wood impregnated with aqueous solution of copper(II) sulfate (a), and exposed to *G. trabeum* for 2 (b), 4 (c), 6 (d) and 12 weeks (e). RG is a relative receiver gain with respect to spectrum a.

the other hand, the intensities of copper EPR decreased. Still, the weak superimposed copper signal was seen in EPR spectra even after twelve weeks of decay (It is resolved after deduction of manganese signal). AAS analysis showed that there is no statistically significant difference between the concentration of copper in treated, unexposed and treated, exposed specimens (Table 2). Thus, we assume that copper was transformed into another form that could not be detected by EPR. *G. trabeum* produce lower amounts of oxalic acid than *A. vaillantii* (Takao 1965) and we believe that the lower production of oxalic acid resulted in slower transformation of copper. If we additionally treated wood impregnated with CuS with the aqueous solution of sulfuric acid, we observed the same overlap in signals as seen during initial stages of decay. On the other hand, treatment with oxalic acid resulted in complete disappearance of the copper(II) EPR signal and the appearance of a strong manganese signal.

Concentrations of copper in nutrient media overgrown by *G. trabeum* (Table 2) were slightly higher than in control or nutrient media overgrown with *A. vaillantii* (0.6 ppm). One of the reasons for this phenomenon might be the described diverse acid production by *G. trabeum*. Lower amounts of oxalic acid, excreted by *G. trabeum* in comparison to *A. vaillantii* means less copper oxalate and at least a portion of Cu(II) must have remained in a form of copper(II) sulfate. Cu in this form is soluble, so the fungus could translocate it to a nutrient medium.

Wood treated with Copper(II) octanoate and ethanolamine

Wood protected with the copper(II) octanoate with ethanolamine (CuE) provided good fungicidal resistance. No mass loss was detected even after 12 weeks of exposure. The first prominent changes in EPR spectra of wood exposed to *G. trabeum* were noticed after 4 weeks of exposure (Fig. 5c). From this spectrum, two superimposed copper EPR signals can be resolved. Copper appears to be present in two different forms. The first form produces a strong EPR signal of immobile copper (either in an insoluble compound or bound to wood) and this signal has the same lineshape as CuE treated unexposed wood ($g_I = 2.062$; $g_{II} = 2.259$; $a_{II} = 16.5$ mT). The other form belongs to a soluble copper. This EPR signal has the same parameters as the EPR signal of an aqueous solution of copper(II) octanoate, oxalic acid and ethanolamine ($g_0 = 2.151$ $a_0 = 6.2$ mT). The same spectrum was also observed during exposure of CuE treated wood to *A. vaillantii*. However, after 12 weeks of exposure the described isotropic signal disappeared and only the anisotropic one remained, as determined by EPR measurements. Concentrations of copper in exposed wood were not significantly different from concentrations of copper in unexposed wood, so the majority of the copper remained in the wood. On the other hand, we detected significantly higher amounts of copper in the nutrient media. The concentration of copper in the nutrient media after 12 weeks of exposure was more than 100 times higher than in a control agar (Table 2). Therefore, we assume that soluble copper was translocated and insoluble copper remained in wood. It should be stressed that copper measured in agar is translocated from five specimens of wood placed above the agar. The extraction from one specimen is five times less and represents 5 % of the initial copper in wood. This translocation was achieved either by active transport, or by diffusion. The latter assumption is more plausible, since fungi moisturize specimens to such an extent that dews

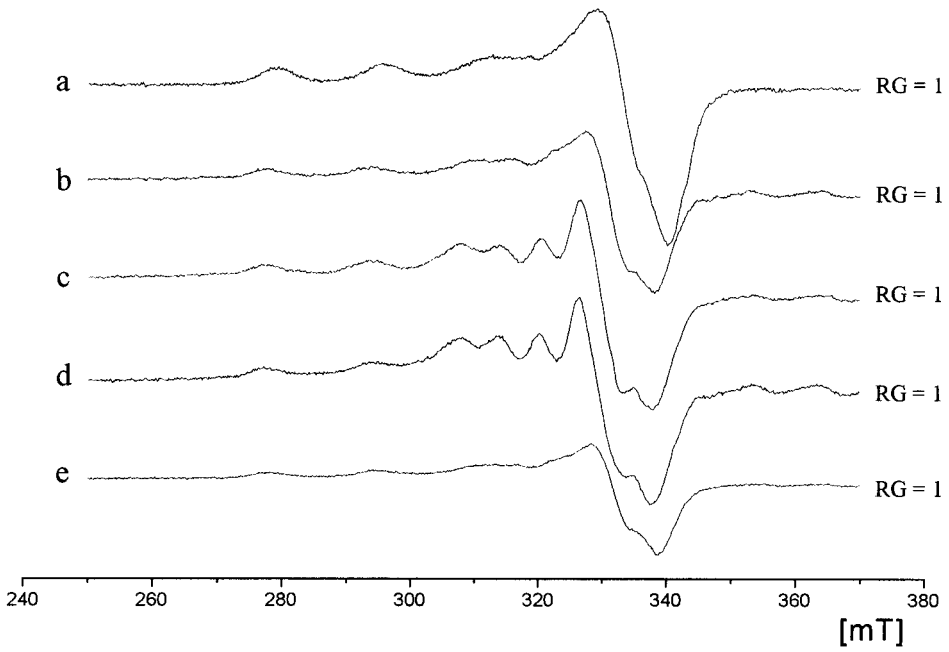

Fig. 5. EPR spectra of wood impregnated with aqueous solution of copper(II) octanoate with ethanolamine (a), and exposed to *G. trabeum* for 2 (b), 4 (c), 6 (d) and 12 weeks (e). RG is a relative receiver gain with respect to spectrum a.

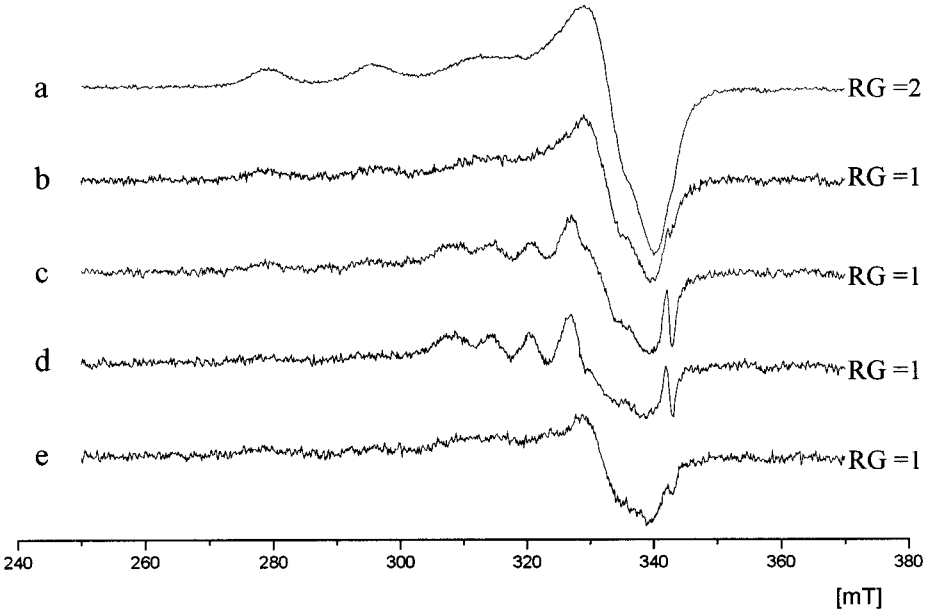

Fig. 6. EPR spectra of wood impregnated with aqueous solution of copper(II) octanoate with ethanolamine (a), and exposed to *T. versicolor* for 2 (b), 4 (c), 6 (d) and 12 weeks (e). RG is a relative receiver gain with respect to spectrum a.

were observed on the surface of the specimens. As the dews were in contact with both specimens and nutrient media, diffusion can take place.

Copper-treated wood exposed to Trametes versicolor

In contrast to previously considered *Antrodia vaillantii* and *Gloeophyllum trabeum*, *Trametes versicolor* is not a brown rot fungus but white rot one. This fungus is widely used for detoxification of some phenolic preservatives (Lamar 1995), and we were interested in how this fungus reacts with copper in wood. Usually this fungus prefers hardwoods (Green and Highley 1997), but in this experiment, we used spruce softwood, with the goal of comparison with our other results, and because softwoods are more often treated with copper-containing preservatives.

Wood treated with Copper(II) sulfate

The first mass loss of wood impregnated with copper(II) sulfate was detected 12 weeks after exposure to *T. versicolor*. At the same time, a weak manganese EPR signal appeared on the spectra, while the EPR signal of free radicals ($g_0 = 2.003$) was observed four weeks before. The anisotropic copper EPR signal was present throughout the experiment, but its intensity decreased slightly over time. We propose that this decrease occurred due to the reaction of copper with small amounts of oxalic acid produced by this fungus. Another reason for this decrease could be scavenging of free radicals formed during white rot lignin degradation. During this reaction, copper(II) is transformed to copper(I), and therefore we could no longer detect the copper EPR signal. Additionally, we noticed a shift of the g_I value from 2.079 for control specimens to 2.087 for specimens exposed to fungus. We assume that this shift results from the increased moisture content of wood (Humar *et al.* 2001 b). No statistically significant changes in copper concentrations in wood treated by CuS and exposed to *Trametes versicolor,* were recorded by AAS (Table 2). However, we detected increased copper content in the nutrient media. The copper concentration increased from 1.1 ppm after 2 weeks to 30.8 ppm after 12 weeks of exposure to *T. versicolor* (Table 2). The reasons for this translocation are similar to the ones described for *G. trabeum.*

Wood treated with Copper(II) octanoate and ethanolamine

T. verscolor reacted with wood impregnated with the copper(II) octanoate with ethanolamine similarly to *G. trabeum*. Mass loss of impregnated wood was not observed even after 12 weeks of exposure (Table 1). The first EPR changes appeared after 4 weeks (Fig. 6c). We noticed copper in two forms, immobile giving anisotropic spectrum and soluble resulting in the isotropic EPR spectrum. At the same time, the EPR signal for free radicals appeared ($g_0 = 2.003$). However, after 12 weeks of exposure, the EPR signal for soluble copper disappeared, and only the less intense signal for anisotropic copper remained (Fig. 6e). Significantly higher amounts of copper were detected in the nutrient media (Table 2), but this copper represents only a minor portion of initial copper, and the most of it remained in the specimens.

Copper-treated wood exposed to Schizophyllum commune

S. commune did not decay the control specimens nor the copper-treated specimens. The only important change caused by this fungus was a previously described shift in the copper g_I value. In copper(II) octanoate with ethanolamine treated wood we did not observe any change at all. In addition, no copper was detected in the nutrient media.

Therefore, we conclude that this fungus did not react with copper in impregnated wood.

Conclusions

Decay of untreated wood caused by brown rot fungi *Antrodia vaillantii* or *Gloeophyllum trabeum* resulted in increased intensities in the manganese EPR signal. The same signal was observed in unexposed specimens acidified with a solution of oxalic or sulfuric acid. Therefore, we believe that the observed increase of manganese signal in decayed wood is a result of acidification caused by fungi or by treatment with acid solution. Intrinsic manganese in wood under acidic conditions converts into a form that can be observed by EPR. During exposure to the white rot fungus *Trametes versicolor*, a manganese signal appeared as well.

Treatment of wood with copper(II) sulfate ($c_{Cu} = 1 \times 10^{-2}$ mol/l) does not preserve it against wood-decaying fungi. According to our results, the oxalic acid excreted by these fungi reacts with copper(II) in the wood to give insoluble, and thus non-toxic, copper oxalate. The pace of this reaction depends on the rate and amounts of excreted oxalic acid. Copper-tolerant *A. vaillantii* produces more oxalic acid than *G. trabeum* or *T. versicolor*. Therefore, *A. vaillantii* more intensively transformed copper(II) sulfate into copper oxalate than other fungi.

In the presence of ethanolamine, the formation of insoluble copper oxalate in wood is blocked, at least during the initial stages of decay. Soluble copper(II) compounds, resolved from the EPR spectra, are fungitoxic, so decay did not occur. However, after a certain degree of exposure, soluble copper is presumably deactivated, mostly by conversion into a non-soluble form and only a minor part of copper is translocated to solid nutrient media.

References

Collett, O. 1992. Comparative tolerance of the brown-rot fungus *Antrodia vaillantii* (DC.: Fr.) Ryv. isolates to copper. Holzforschung *46*, 293–298.

Da Costa, E. W. B. 1959. Abnormal resistance of *Poria vaillantii* (D. C. ex Fr.) Cke. strains to copper-chrome-arsenate wood preservatives. Nature *183*, 910–911.

Dobbs, A. J. 1974. G-factors of free radicals in fluid solution. Electron Spin Resonance Spec. Period. Rep. *2*, 281–294.

Edwards, H. G. M., D. W. Farwell, S. J. Rose and D. N. Smith. 1991. Vibration of copper(II) oxalate dihydrate, $CuC_2O_4 \times 2H_2O$, and dipotassium bis-oxalato copper(II) tetrahydrate, $K_2Cu(C_2O_4) \times 4\ H_2O$. J. Mol. Struct. *249*, 233–243.

European Committee for Standardization. 1989. Wood preservatives; Determination of the toxic values against wood destroying basidiomycetes cultured an agar medium, EN 113. Brussels, 14.

Felton, C. C. and R. C. DeGroot. 1996. The recycling potential of preservative-treated wood. Forest Products J. *46*, 37–46.

Gadd, G. M. 1993. Interactions of fungi with toxic metals. New Phytologist *124*, 25–60.

Green, III F. and T. L. Highley. 1997. Mechanism of brown-rot decay: Paradigm or paradox. Int. Biodeterior Biodegradation *39*, 113–124.

Green, F., M. J. Larsen, J. E. Winandy and T. L. Highley. 1991. Role of oxalic acid in incipient brown-rot decay. Material und Organismen *26*, 191–213.

Harańczyk, H. 1999. The investigation of hydration processes in horse chestnut (*Aesculus hippocastanum*, L.) and pine (*Pinus silvestris*, L.) bark and bast using proton magnetic relaxation. Holzforschung *53*, 299–310.

Hirt, R. R. 1949. An isolate of *Poria xantha* on media containing copper. Phytopathologist *39*, 31–36.

Hughes, A. S., R. J. Murphy, J. F. Gibson and J. A. Cornfield. 1994. Electron paramagnetic resonance (EPR) spectroscopic analysis of copper based preservatives in *Pinus sylvestris*. Holzforschung *48*, 91–98.

Humar, M., M. Petrič and F. Pohleven. 2001a. Changes of pH of impregnated wood during exposure to wood-rotting fungi. Holz Roh- Werkstoff *59*, 288–293.

Humar, M., M. Petrič and M. Šentjurc. 2001b. Influence of moisture content on EPR parameters of copper in impregnated wood. Holz Roh- Werkstoff *59*, 254–255.

Illman, B. L. and T. L. Highley. 1996. Fungal degradation of wood treated with metal-based preservatives: 1. Fungal tolerance. The International Research Group on Wood Preservation; IRG/WP 96–10163, 7.

Illman, B. L., D. C. Meinholtz and T. L. Highley. 1988. Generation of hydroxyl radical by the brown-rot fungus *Postia placenta*. The International Research Group on Wood Preservation; IRG/WP 88–1360.

Jellison, J., J. Connolly, B. Goodell, B. Doyle, B. Illman, F. Fekete and A. Ostrfsky. 1997. The role of cations in the biodegradation of wood by brown rot fungi. Int. Biodeterior Biodegradation *39*, 165–179.

Kirk, T. K. and D. Cullen. 1998. Enzymology and molecular genetics of wood degradation by white-rot fungi. *In:* Environmental Friendly Technologies for the Pulp and Paper Industry. Eds. R. A. Young, M. Akhtar. John Wiley & Sons, New York. pp. 273–307.

Kuroda, Y. and M. Kubo. 1960. Infrared absorptions of the copper(II) salts of some ω – dicarboxylic acids, J. of Phys. Chem. *64*, 759–762.

Lamar, R. T. 1995. Use of wood-decay fungi for disposal of PCP-treated wood. The International Research Group on Wood Preservation; IRG/WP 95-50040, 8.

Murphy, R. J. and J. F. Levy. 1983. Production of copper oxalate by some copper tolerant fungi. Trans. British Mycology Soc. *81*, 165–168.

Petrič, M, F. Pohleven, U. Schoknecht and M. Šentjurc. Electron Paramagnetic Resonance (EPR) as a method for investigations of microbiological decay process of impregnated wood. unpublished results.

Pohleven, F., M. Šentjurc, M. Petrič and F. Dagarin. 1994. Investigation of ammoniacal copper(II) octanoate in aqueous solutions and its determination in impregnated wood. Holzforschung *48*, 371–374.

Pohleven, F., Š. Breznikar, P. Kalan and M. Petrič. 1999. Determination of absorption, accumulation and transport of copper in mycelium of some wood decay fungi. The International Research Group on Wood Preservation; IRG/WP 99–10323; 9.

Qian, Y. and B. Goodell. 2000. The effect of low molecular weight chelators on iron chelation and free radical generation as studied by ESR measurement. The International Research Group on Wood Preservation; IRG/WP 00–10367, 11.

Raspor, P., S. Smole-Možina, J. Podjavoršek, F. Pohleven, N. Gogala, F. V. Nekrep, I. Rogelj and J. Hacin. 1995. ZIM: zbirka industrijskih mikroorganizmov. Katalog biokultur, Biotehniška fakulteta, Katedra za biotehnologijo, Ljubljana, 98.

Richardson, H. W. 1997. Handbook of Copper Compounds and Applications. M. Dekker, New York. pp. 93–122.

Sharp, R. F. 1975. The interactions of fungi, wood preservative and wood. The movement of copper by hyphae as observed with a model technique. Wood Science and Techn. *9*, 99–111.

Skoog, D. A., D. M. West and F. J. Holler. 1992. Fundamentals of Analytical Chemistry. Saunders, Fort Worth, USA. pp. 118–143.

Srivastava, P. C., S. Banerjee and B. K. Banerjee. 1980. Magnetic & spectroscopic behaviour of some copper oxalate complexes. Indian J. Chem. *19A*, 77–79.

Sutter, H. P., E. B. G. Jones and O. Wälchli. 1983. The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.) Fr. Material und Organismen *18*, 241–262.

Takao, S. 1965. Organic acid production by basidiomycetes, I. Screening of acid-producing strains. Appl. Microbio. *13*, 732–737.

Tsunoda, K., K. Nagashima, M. Takahashi. 1997. High tolerance of wood-destroying brown-rot fungi to copper-based fungicides. Material und Organismen *31*, 31–44.

White, C., J. A. Sayer and G. M. Gadd. 1997. Microbial solubilization and immobilization of toxic metals: Key biogeochemical processes for treatment of contamination. FEMS Microbiology Reviews *20*, 503–516.

Woodward, B. and R. De Groot. 1999. Tolerance of *Wolfiporia cocos* isolated to copper in agar media. Forest Products J. *49*, 87–94.

Zabel, R. A. 1954. Variations in preservative tolerance of wood-destroying fungi. Forest Product Research Soc. J. *4*, 166–169.

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