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Influence of a nitrogen supplement on the growth of wood decay fungi and decay of wood

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Abstract

Bioremediation processes require cheap and effective nutrient sources which contain significant amounts of nitrogen, e.g. corn steep liquor (CSL). In order to elucidate fungal copper tolerance in a nitrogen-rich environment, experiments were performed on a nutrient medium and with wood. CSL was added to nutrient medium containing different copper concentrations and to Norway spruce (*Picea abies*) wood specimens impregnated with a commercial copper-based preservative (CCB). Sterilized CCB-impregnated and control CSL-supplemented specimens were exposed to copper-tolerant (*Antrodia vaillantii*, *Leucogyrophana pinastri*) and copper-sensitive (*Postia placenta*, *Gloeophyllum trabeum, Trametes versicolor* and *Hypoxylon fragiforme*) fungal species according to the mini-block procedure. Additionally, nutrient media containing CSL and copper(II) sulphate of different concentrations were inoculated with the same fungi and the growth of the fungal hyphae was visually estimated. The results of both experiments showed that CSL increases the ability of the copper-sensitive brown- and white-rot fungi to grow on copper-containing substrates. CSL inhibited growth of the copper-tolerant fungi on nutrient medium containing copper and decreased decay of CCB-preserved wood. It is believed that the reason for changed copper tolerance originates in copper-tolerant fungi producing less oxalic acid in the presence of high concentrations of nitrogen in the growth environment.

Keywords: Copper-tolerant fungi; Wood decay fungi; Bioremediation; Waste-treated wood; Corn steep liquor; Nitrogen-rich substrate

1. Introduction

Copper compounds were one of the most important biocides in wood preservatives for almost 200 yr. In this period, wood decay fungi developed mechanisms that allowed them to survive on copper-treated wood. Fungi that are able to grow on the substrate of copper concentrations higher than 1.6 mmol L^{-1} are considered to be copper tolerant (Gadd, 1993). The phenomenon of copper tolerance has been known for more than 55 yr (Hirt, 1949), but has become more important during the past 20 yr. Despite this fact, the exact mechanism of copper tolerance and copper toxicity are not completely understood. Fungal Cu-tolerance is linked to oxalic acid excretion (Clausen and Green, 2003). This acid is involved in wood-rotting fungal-mediated processes of ligno-cellulose degradation, particularly in the initial phases of wood colonization. Predominantly brown-rot fungi excrete significant amounts of oxalic acid that react with copper in wood to form copper oxalate (Jarosz-Wilkolazka and Gadd, 2003; Humar et al., 2004). As copper oxalate is insoluble, it is less toxic to wood decay fungi. However, the phenomenon of copper tolerance cannot be ascribed to the formation of insoluble copper oxalate alone, as it was proved that even soluble copper is less fungitoxic in acidic substrates (pH 1–3) than in slightly acidic or even neutral conditions (Humar et al., 2005).

Copper-tolerant organisms are of scientific interest from two different points of view. Firstly, if we know the exact mechanisms of tolerance, we could develop new

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preservatives that are more efficient. On the other hand, we could use copper-tolerant organisms for biorecycling of copper-containing waste wood through bioremediation and bioconversion (Humar et al., 2004).

Biotechnological processes, such as bioremediation or bioconversion of waste CCA-treated wood, usually require an inexpensive nutrient source. In this experiment, relatively inexpensive and readily available corn steep liquor (CSL) was used. It comes from the corn wet milling process; it is already used in several biotechnological processes (Akhtar et al., 1997). CSL is a viscous concentrate of corn solubles, rich in proteins and peptides (20–25%), amino acids, lactic acid (7–9%), minerals, vitamins and other growth stimulants, with pH approx. 4. It contains 50–60% solids. Approx. 90% of the nitrogen present in CSL is amino nitrogen, >95% being present in peptides and <5% in free amino acids. The total nitrogen content of CSL, which varies from batch to batch, is 3–5% (Schroeder, 1997).

Fungi, like other organisms, require substantial amounts of nitrogen for synthesis of proteins and other cell constituents (Zabel and Morrell, 1992). However, we were interested in how the presence of a rich nitrogen environment influences the growth and decay abilities of copper-tolerant and copper-sensitive fungi in the presence of copper-based wood preservatives. The influence of nitrogen on fungal growth is interesting from a wood preservation perspective as well, since several new wood preservatives contain amines (Cao and Kamdem, 2004).

2. Materials and methods

2.1. Test on nutrient medium

Diluted solutions of copper (II) sulphate were added to 10 ml cooling sterilized potato dextrose agar (PDA, Difco) to achieve the following final copper concentrations of 1×10^{-3}, 5×10^{-3}, 1×10^{-2} and 2.5×10^{-2} mol L^{-1}. Medium without copper was used for controls. In order to determine the influence of CSL on copper toxicity, appropriate amounts of CSL were added to selected tubes to achieve a final concentration of 1 or 5%. The solidified growth media were inoculated with 0.7 cm diameter pieces of mycelium of the wood decay fungi listed in Table 1. *A. vaillantii* and *L. pinastri* strains used were shown to be copper-tolerant previously (Humar et al., 2001; Pohleven et al., 2002). The *L. pinastri* culture was kindly provided by Sam Amartey of the Forest Products Research Centre, Buckinghamshire Chilterns University College, High Wycombe, United Kingdom. The other cultures were obtained from our fungal culture collection. The tubes were then incubated in a growth chamber at 25 °C and fungal growth was assessed visually after one week of exposure and compared with growth of controls. The experiment was performed with five replicate tubes per treatment.

Fungicidal activity was estimated by fungal growth retardation, using the following visually determined marks:

0 mycelium growth more intense than control,
1 normal growth, insignificant retardation (area of colony ⩾90% of area of controls),
2 visible signs of retardation (colony <90% and ⩾60% of controls),
3 pronounced retardation (colony <60% and ⩾25% of controls),
4 very marked retardation (colony <25% of controls),
5 no growth.

2.2. Mini-block procedure

Norway spruce (*Picea abies*) samples ($0.5\times10\times30$ mm) were vacuum impregnated with 1% or 5% CCB solution according to the EN 113 procedure (European Committee for Standardization (ECS), 1989). The treatment resulted in a preservative uptake of about 4 kg/m^3 for specimens impregnated with 1% CCB solution and 19 kg m^{-3} for those impregnated with 5% CCB. The samples were then conditioned for four weeks: the first two weeks in closed chambers, the third week in half closed chambers and the fourth week in open chambers. The conditioned samples were then oven dried (75 °C) for three days in order to ensure complete reduction of chromium. Following

Table 1
Characteristics of wood decay fungi used in tests

Fungi	Origin	Estimated Cu tolerance	Type of rot
Antrodia vaillantii	University of Ljubljana ZIM L037	Cu-tolerant, 1	Brown
Leucogyrophana pinastri	Buckinghamshire Chilterns University College UK	Cu-tolerant, 2	Brown
Postia placenta	University of Ljubljana ZIM L033	Cu-sensitive/Cu-tolerant, 3	Brown
Gloeophyllum trabeum	University of Ljubljana ZIM L018	Cu-sensitive, 5	Brown
Trametes versicolor	University of Ljubljana ZIM L057	Cu-sensitive, 5	White
Hypoxylon fragiforme	University of Ljubljana ZIM L108	Cu-sensitive, 5	White

Copper tolerance is rated according to Pohleven et al. (2002): 1 = greatest copper tolerance; 5 = greatest copper sensitivity.

conditioning, the samples were leached according to the EN 84 procedure for 14 days (European Committee for Standardization (ECS), 1994). Afterwards, the samples were oven dried (103 °C), and their initial masses were determined. Two-thirds of the dry impregnated and control specimens were left to moisturize again before vacuum treatment with 1 or 5% aqueous solution of corn steep liquor (CSL) (Sigma). The vacuum treatment resulted in an uptake of solution of 270 kg m^{-3}. The CSL-immersed specimens were then dried for one week prior to exposure to fungi. One third of the specimens were treated with distilled water instead of CSL to serve as controls.

Conditioned and steam-sterilized specimens were then exposed to six different fungal species (Table 1) using the mini block procedure (Pohleven et al., 2000). Cultures were grown and maintained on a 3.9% potato dextrose agar medium (PDA, Difco). Petri dishes with PDA were inoculated with small pieces of fungal mycelium. Three treated samples and one untreated wood sample were placed on a sterilized plastic grid in each inoculated Petri dish and exposed to fungal decay for eight weeks in the growth chamber (25 °C, RH 75%). The final oven dry masses of individual specimen was then determined and the respective losses in mass calculated.

2.3. Electron paramagnetic resonance (EPR) spectroscopy

In order to determine the interactions between chromium and copper in impregnated wood with CSL, EPR spectra of CSL-treated and untreated specimens were measured. The spectra of the control and decayed samples were recorded at room temperature using 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 ($1 \times 1 \times 40$ mm) were cut from each wood sample and separately inserted into the resonator. Where possible, the various components of EPR parameters (tensor g, and hyperfine splitting tensor A) were determined directly from the spectra for the respective paramagnetic species.

2.4. Nitrogen content

Nitrogen and carbon content in the specimens was determined in ground conditioned specimens. For that purpose, 0.2 g dry sample was combusted in the oxygen atmosphere at 1350 °C in a LECO 2000-CNS analyser. The nitrogen content of triplicate sub-samples was determined in the thermo-combustion cell.

3. Results

The copper-tolerant fungi *Leucogyrophana pinastri* and *Antrodia vaillantii* were able to grow even on the nutrient medium with the highest copper concentration (2.5×10^{-2} mol L^{-1}). In comparison, the white-rot fungi, *Trametes versicolor* and *Hypoxylon fragiforme,* did not grow on the medium containing only 4% (1.0×10^{-3} mol L^{-1}) of the latter concentration. The highest sensitivity to copper was exhibited by the brown-rot fungus *Gloeophyllum trabeum* (Fig. 1).

The presence of CSL in the medium without copper did not appear to have a significant influence on the fungal growth. The brown-rot fungi *A. vaillantii, L. pinastri* and *G. trabeum* were not affected by the presence of CSL. The presence of CSL slightly increased the fungal growth of *P. placenta* and *H. fragiforme*, and also seemed to inhibit the growth of *T. versicolor* (Fig. 1) to some extent.

The CSL-supplemented nutrient medium did not have a uniform effect on the copper toxicity. The presence of CSL in nutrient medium decreased the ability of the copper-tolerant fungi to grow on the copper-containing medium. For example, the fungus *L. pinastri* could normally survive on the medium containing the highest copper concentration (2.5×10^{-2} mol L^{-1}) but not in the presence of 5% CSL. A similar but less prominent influence of CSL was observed with the copper-tolerant fungi *A. vaillantii* and *P. placenta* as well (Fig. 1). Conversely, the presence of CSL positively influenced the ability of the white-rot fungi, *T. versicolor* and *H. fragiforme,* and the copper-sensitive brown-rot species *G. trabeum,* to resist the presence of copper in the nutrient medium. In the presence of 5% CSL, this brown-rot fungus grew at twice the copper concentration than it did in nutrient medium without CSL (Fig. 1).

The results on the nutrient medium were not always comparable with the results from the wood, as in the nutrient medium all the nutrients required were readily available to the fungi. Additionally, wood fungi have to expend more energy and use very sophisticated mechanisms to colonize the wood in order to survive. Thus, we were interested in finding out how the presence of CSL in preserved wood influenced the ability of fungi to decay wood. Nitrogen content in wood increased from 0.096% in untreated wood, to 0.176 in specimens treated with 1% CSL solution and to 0.325% those treated with 5% CSL solution. On the other hand we did not observe statistically significant difference in carbon concentration after CSL treatment (Not shown).

Mass losses of the unimpregnated control specimens were comparable with other experiments and indicated that all the fungi were fully active, and the experimental procedure was satisfactory (Humar et al., 2001). The unimpregnated specimens that were supplemented with

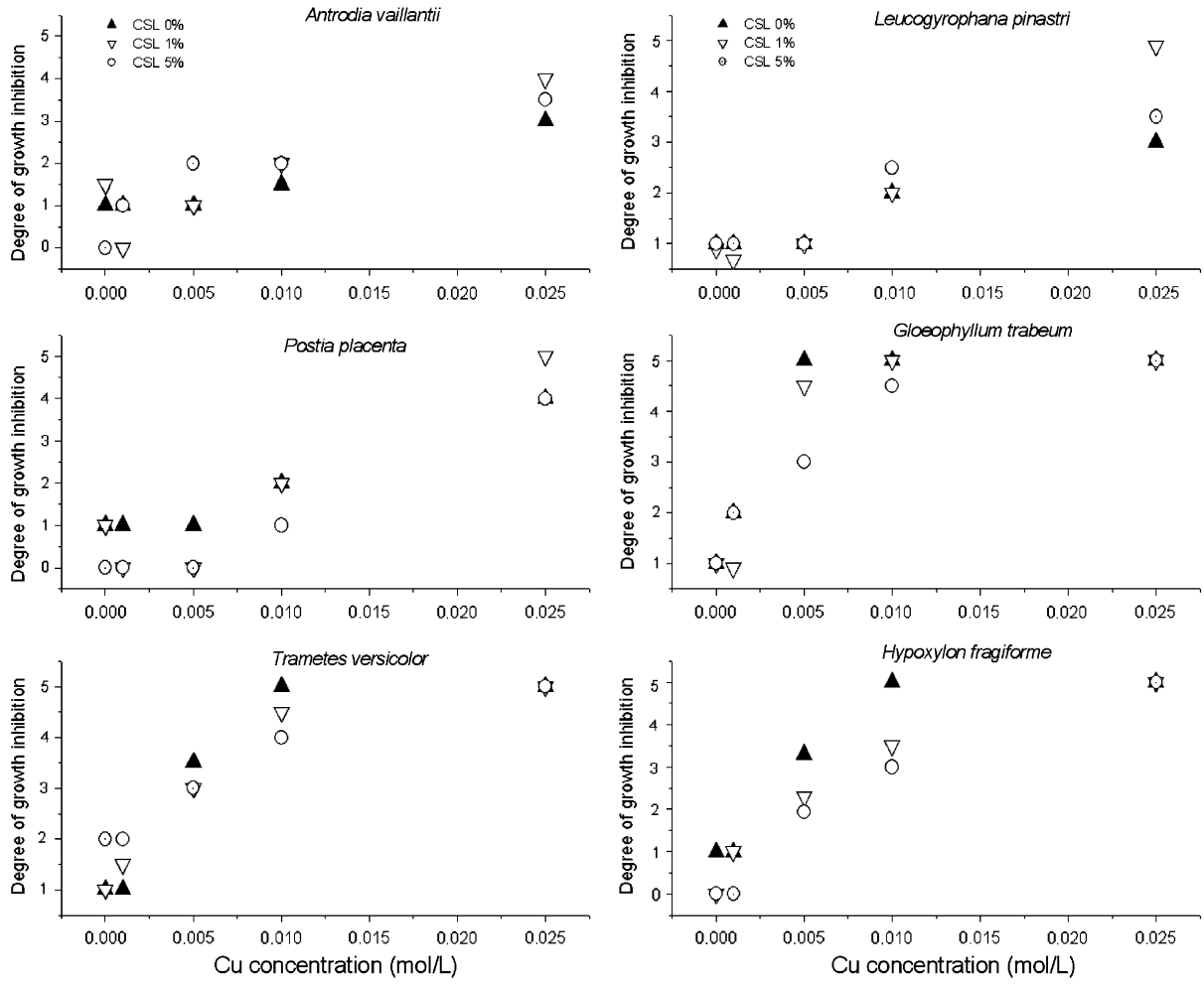

Fig. 1. Influence of addition of CSL and copper sulphate to nutrient medium on growth of white- and brown-rot fungi. Growth was estimated according to the scale described in Section 2.1.

CSL were significantly less decayed than samples without CSL, with the exception of *A. vaillantii*. For this fungus, the control samples showed an average mass loss of 18.4% while the specimens immersed in 5% CSL solution lost an average of 24.3% of their mass. On the other hand, *G. trabeum* decayed 56.6% of the control specimens without CSL after 8 weeks which was more than 12 times greater than the average mass loss in wood supplemented with 5% CSL (Table 2). The negative influence of CSL on brown-rot fungal growth was expected, since brown-rot fungi require less nitrogen for their growth than do white-rot species (Zabel and Morrell, 1992). Thus, the negative influence of CSL on the growth and decay of the white-rot fungi *T. versicolor* and *H. fragiforme* is rather surprising (Table 2).

The results obtained on the CCB-treated wood are comparable with the initial screening tests. CSL had a negative influence on decay abilities of the copper-tolerant fungi (Fig. 1). For instance, *A. vaillantii* decayed 5.5% of the substance of specimens impregnated with 5% CCB solution, while a mass loss of only 1% was detected for specimens immersed in 1 or 5% CSL prior to exposure. Similar results were determined with specimens exposed to *L. pinastri* (Table 2). However, copper-sensitive *G. trabeum* did not decay any CCB-treated specimens whether treated with CSL or not. This result reflects the high copper sensitivity of this fungus (Pohleven et al., 2002). On the other hand, CSL enabled the white-rot fungi *T. versicolor* and *H. fragiforme* to decay CCB-treated specimens to a significantly higher extent than controls. In the presence of 1% CSL, *H. fragiforme* decayed 5.2% of the specimen treated with 5% CCB solution, while there was no significant mass loss (0.6%) in unsupplemented controls (Table 2).

The properties of the paramagnetic active molecules, such as free radicals, copper(II), chromium(III), chromium(V) can be determined using EPR spectroscopy. The EPR spectra of the CCB-treated specimens that were immersed in CSL solutions 1 or 5% have parameters and shapes of spectra comparable to those that were immersed in distilled water only. From those

Table 2
Loss in dry mass of unimpregnated and CCB-impregnated mini-blocks immersed in 1% or 5% aqueous solution of CSL, after exposure to wood decay fungi *Antrodia vaillantii* (Av), *Leucogyrophana pinastri* (Lp), *Postia placenta* (Pp), *Gloeophyllum trabeum* (Gt), *Trametes versicolor* (Tv) and *Hypoxylon fragiforme* (Hf) for eight weeks

CCB conc. (%)	CSL conc. (%)	Fungus					
		Av	Lp	Pp	Gt	Tv	Hf
		Mass loss (%)					
0	0	18.4 (3.4)	17.3 (3.5)	45.8 (4.0)	56.6 (5.4)	21.8 (4.2)	31.6 (5.8)
	1	18.5 (4.6)	9.4 (2.1)	50.4 (5.1)	19.0 (6.3)	18.8 (3.7)	20.7 (3.5)
	5	24.3 (3.0)	6.8 (2.4)	26.8 (4.4)	4.6 (3.9)	12.3 (2.1)	19.4 (3.4)
1	0	31.7 (3.0)	20.8 (3.2)	30.9 (6.8)	0.9 (0.2)	0.5 (0.1)	0.1 (0.3)
	1	29.1 (3.1)	17.4 (2.9)	34.7 (3.2)	0.5 (0.2)	0.5 (0.1)	0.2 (0.2)
	5	29.3 (2.9)	13.9 (1.8)	33.6 (2.6)	−1.1 (0.3)	2.1 (0.1)	4.1 (1.0)
5	0	5.5 (1.0)	2.9 (0.9)	−0.6 (1.0)	0.8 (0.2)	0.6 (0.2)	0.6 (0.2)
	1	1.1 (0.9)	1.5 (0.5)	−0.9 (0.4)	0.3 (0.0)	3.1 (0.3)	5.2 (2.1)
	5	1.0 (0.4)	1.1 (0.6)	−1.0 (0.4)	−1.7 (1.0)	1.6 (0.6)	1.9 (0.1)

Standard deviations are given in the parenthesis.

spectra, two different EPR signals can be distinguished: Cr(III) EPR signal ($g = 1.97$, line width = 54 mT) and Cu(II) EPR signal ($g_{\perp} = 2.077$, $g_{II} = 2.322$, $A_{\perp} = 13.4$ mT). Similar parameters (signals) for CCB-treated wood have been reported in the literature as well (Hughes et al., 1994; Humar et al., 2004). These parameters indicate that there were no new complexes formed between copper or chromium and CSL.

4. Discussion

EPR spectroscopy confirmed that changes in growth of fungi on CSL supplemented wood were not caused by chemical reaction between CSL and the copper/chromium compounds in impregnated wood. As mentioned in the Introduction, CSL consists mainly of proteins, lactic acid, minerals, vitamins and other growth stimulants. In our previous investigations it was shown, that lactic acid, like most other organic acids, increased the ability of wood decay fungi to resist copper. However, the concentration of lactic acid in diluted CSL solution was relatively low (0.4–0.07%). At such low acid content there was no appreciable influence of organic acids on copper toxicity (Humar et al., 2005). As wood is a mixture of various carbohydrates, it can be presumed that addition of other carbohydrates present in CSL should not affect the fungal growth to a significant extent. Therefore, it can be considered that results of the screening test and mass loss experiments reflect primarily the influence of increased nitrogen concentration in the medium on the copper toxicity.

CSL has always been considered to be a cheap and effective nutrient source (Akhtar et al., 1997). However, our results indicate that even 1% CSL aqueous solution decreases the ability of both white- and brown-rot fungi to decay and sometimes even to grow on the wood substrates (Fig. 1, Table 2). Additionally, CSL decreased the growth of the copper-tolerant fungi on copper-preserved wood as well (Table 2), which minimizes the potential for use of CSL in the bioremediation process of waste CCA-treated wood. On the other hand, from the wood preservative perspective this result can be regarded as promising and helps to explain the greater effectiveness of the copper/ethanolamine wood preservatives towards copper-tolerant fungi (Humar et al., 2001).

The reason for increased copper effectiveness against copper-tolerant fungi in the presence of high nitrogen concentrations may be explained by changes in oxalic acid excretion. Oxalic acid plays a very important role in the phenomenon of copper tolerance (Humar et al., 2001; Jarosz-Wilkolazka and Gadd, 2003). Akamatsu and co-workers (1994) compared the amounts of the oxalate accumulated by 13 brown-rot and 11 white-rot fungi growing on low nitrogen and high nitrogen media. Copper-tolerant fungi produced significantly less oxalic acid in high nitrogen media than in low nitrogen media. On the other hand, the white-rot fungus *T. versicolor* produced more oxalic acid in high nitrogen media and less acid in low nitrogen media. *G. trabeum* is known as one of the brown-rot fungi that produce the lowest amounts of oxalic acid both in media of low or high nitrogen content (Takao, 1965; Akamatsu et al., 1994). Thus, copper-tolerant fungi that excrete less oxalic acid on nitrogen-rich media caused lower mass losses on CSL-supplemented CCB-treated specimens. White-rot fungi were stimulated to excrete more oxalic acid in the presence of CSL, which was reflected in higher mass losses of the CCB-treated wood supplemented with CSL

(Table 2). In addition, growth of the white-rot fungi on CSL containing nutrient medium was less inhibited (Fig. 1). Finally, *G. trabeum,* a fungus that does not excrete oxalic acid in media regardless of nitrogen concentration (Takao, 1965), did not decay any CCB-treated specimens (Table 2).

5. Conclusions

The presence of corn steep liquor increases the ability of the copper-sensitive brown-rot and white-rot fungi to grow on copper-containing substrates. CSL decreased the growth of the copper-tolerant fungi on nutrient medium containing copper and reduced decay of CCB-preserved wood as well. We believe that the reason for decreased copper tolerance originates in the fact that copper-tolerant fungi produce less oxalic acid in the presence of high nitrogen concentrations. In contrast, addition of nitrogen stimulates the copper-sensitive white-rot species *T. versicolor* and *H. fragiforme* to excrete more oxalic acid and thus increase the abilities of those fungi to resist copper in the substrate.

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