Biodegradation (2009) 20:135–142
DOI 10.1007/s10532-008-9207-1

ORIGINAL PAPER

Tolerance to wood preservatives by copper-tolerant wood-rot fungi native to south-central Chile

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Received: 16 January 2008 / Accepted: 8 July 2008 / Published online: 25 July 2008

Abstract Understanding the effect of heavy metals and wood preservatives on the growth of wood-rot fungi native to a certain region may improve reliability in determining the effectiveness of antifungal products, particularly when dealing with new formulations. In this investigation, strains of copper-tolerant wood-rot fungi native to south-central Chile were evaluated against two preservatives: commercial chromated copper arsenate type C (CCA-C) and a new formulation with boron and silicon (BS). Thirteen native strains, mainly white-rot fungi, were selected for their high growth rates in solid medium containing 3 mM of copper. A short-term test was then carried out, consisting of adding cellulose disks impregnated with different concentrations of preservatives to solid culture media inoculated with selected copper tolerant strains. There was a great variability in interspecific and intraspecific responses to the presence of copper and preservatives in culture media. Among the native and commercial strains evaluated, the white-rot fungi *Trametes versicolor* 38 and mainly *Ganoderma australe* 100 were notable for their tolerance to all the CCA-C and BS concentrations. The brown-rot fungus *Wolfiporia cocos*, used as reference strain, showed a high tolerance to CCA-C, but not to BS preservative. *T. versicolor* 38 and *G. australe* 100 were selected for subsequent studies on preserved wood degradation.

Keywords Boron–silicon preservative · Chromated copper arsenate preservative · Copper-tolerant fungi · Native fungi · Wood preservatives

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Introduction

A variety of wood-rot (brown-rot or white-rot) and wood stain fungi have been reported to be tolerant to heavy metals (Baldrian 2003; Green and Clausen 2005; Guillén and Machuca 2008). Wood-rot fungi tolerant to copper (Cu) are of great interest with respect to research into the bioremediation of soils or industrial effluents polluted with metals (Baldrian 2003) and in the bioremediation of woods treated with copper-based preservatives that have served their purpose and have been discarded as waste (Humar et al. 2004, 2006; Illman and Yang 2004). These tolerant fungi are also particularly interesting for investigations related to new wood protectants since one of the many requirements for these systems is its effectiveness against metal-tolerant fungal species (Przewloka 2004; Schultz et al 2005). When new preservative formulations are developed, various laboratory tests using specific fungal species are necessary to evaluate efficiency prior to the preservative being marketed (Przewloka 2004; American Wood Preservers' Association (AWPA) 2004). In this context, research has been more oriented towards brown-rot fungi than white-rot because the brown-rot fungi in general present a greater tolerance to copper-based preservatives and they are much more important in structural conifers which dominate the building market (De Groot and Woodward 1999; Illman and Yang 2004). In most cases, the tests for new wood preservatives consist of directly adding the preservative to a solid medium ("agar-plate" test) or depositing impregnated wood blocks on solid medium ("agar-block" test) or on soil ("soil-block" test). Each of these tests has both advantages and limitations: most of them require extremely long periods of time, particularly when they involve the use of wood. The complexity of many preservative formulations makes it difficult to add them directly to a culture medium without incurring insolubility problems (Archer et al. 1995; American Wood Preservers' Association (AWPA) 2004).

Along with the research into new preservative formulations, it is also important to know the native fungal diversity normally confronting non-preserved and preserved woods in a certain region, such as that selected for this study, whose industrial activities are predominantly forestry and lumber. For this reason, various strains of wood-rot fungi native to a region of south-central Chile, were chosen for their copper tolerance in solid medium and then evaluated through a fast and simple test for their resistance to two wood preservatives of differing composition. The preservatives evaluated are of the well-known commercial chromated copper arsenate type C (CCA-C) and to a new preservative developed recently in Chile and based on boron and silicon (BS).

Materials and methods

Native fungal strains

Wood-rot basidiomas were collected from decayed wood in different ecosystems from the Provinces of Concepción and Bio-Bio, located in the VIIIth Region, 514 km south of Santiago, Chile, between May and October, 2004 and 2005. Small fragments of the basidiomas were excised under aseptic conditions to obtain mycelial cultures on a 2% (m v^{-1}) malt extract agar (MEA), incubated at 24 ± 1°C, in the dark for the time needed to obtain pure fungal colonies. Identification of basidiomas was made by using different taxonomic keys (Wright and Deschamps 1972; Ryvarden 1987, 1991; Bernicchia 2005; Rajchenberg 2006) and by comparisons with the material deposited in herbaria.

Copper-tolerance test

Copper tolerance of the native fungal strains was evaluated on 2% MEA, with pH adjusted to 4.5 and a 3 mM concentration of copper, added to the medium as solid salt ($CuSO_4 \cdot 5H_2O$), after sterilization and when the medium reached an appropriate temperature. The plates were incubated with inoculi 7 mm in diameter, in the dark at 24 ± 1°C, for a maximum of two months, a period in which the copper tolerance was determined as the growth rate by measuring the radius of the colony at time intervals. The radii of the colonies over time were used to plot growth curves that were adjusted using a linear regression equation to obtain the average growth rate in millimeters per day (mm day^{-1}) (Chapman et al. 1990; Guillén and Machuca 2008). The test was performed in triplicate for each isolate. Each strain was also evaluated on non-copper amended media.

Preservative solutions

cA commercial solution of CCA-C and the BS solution were evaluated at different concentrations (Table 1) to assess their effect on the growth of copper-tolerant fungal strains in solid medium. The concentrations tested were those used commercially for wood impregnation. CCA-C preservative contains 18.5% CuO, 47.5% CrO_3, and 34% As_2O_5 as specified by American Wood Preservers' Association (AWPA) (1999). The water-based BS preservative, whose general formula is $(Na_2O)m\ (SiO_2)n(B_2O_3)p$, is a new preservative developed in Chile (Navarrete 2004; Escobar 2005) that is starting to be applied for commercial wood impregnation by Stonewood®. Both preservatives were kindly donated by Preserva Ltda.

Table 1 Wood preservative solutions used in in vitro tolerance tests in solid medium

Preservative solution	Composition	Concentration % (m/m)
CCA (Type C)	Chromated copper arsenate	1; 1.5 and 2.4
BS (I)	0.2% Boron and 2.5% Silicon	2.7
BS (II)	0.6% Boron and 2.5% Silicon	3.1
BS (III)	0.2% Boron and 5% Silicon	5.2
BS (IV)	0.6% Boron and 5% Silicon	6.1

Effect of preservatives on in vitro growth

Preservative effectiveness was only evaluated on native fungal strains that were tolerant to 3 mM Cu. *Wolfiporia cocos* ATCC 62778 and *Gloeophyllum trabeum* ATCC 11539, from the brown-rot fungi (BRF) group, and *Trametes versicolor* DEBIQ01 and *Ganoderma australe* A464, from the white-rot fungi (WRF) group were used as reference strains (Guillén and Machuca 2008). The stock cultures of all the strains, native and reference, were prepared and maintained in a 2% MEA medium.

The effect of CCA-C and BS preservatives on the growth of the different fungal strains was evaluated using a modification of the method proposed by Alleman et al. (1993) to select fungi resistant to toxic substances using cellulose antibiotic assay disks. The modification made it possible to quantitatively evaluate the effect of preservative concentration on growth of the various fungal strains by determining the radial growth rate (mm day^{-1}). The Petri dishes containing 2% MEA medium (pH 4.5) were inoculated at the edge with an agar-mycelium disk and incubated at 24 ± 1°C in the dark until the colonies reached a radius of 2–3 cm. A cellulose disk (13 mm diameter) impregnated with a certain concentration of preservative aseptically was placed at the opposite edge of the dish. The dishes were incubated under the same conditions previously described, for 8 days. Radial growth rate (mm day^{-1}) was evaluated as mentioned above, before and after adding the impregnated cellulose disks with the preservatives. Each fungal strain was inoculated in triplicate with the same concentration of preservative. Disks impregnated with different CCA-C and BS concentrations were placed on sterile MEA medium (without fungi) and incubated for 8 days in order to evaluate visually the diffusibility of the preservative solutions.

Statistical analyses

In order to evaluate the effect of the copper and wood preservatives on the growth rate of each fungal strain, a unifactorial experimental design was applied completely at random using the STATISTICA program (Version 6 2001, Statsoft, Tulsa). Variance homogeneity was verified for the variables. The significant differences were assessed using Tukey's test at $P < 0.05$.

Results

Copper-tolerant strains

Seventy-one strains of pure culture basidiomycetes were obtained from the basidiomas collected in the field, of which 48 grew in 3 mM Cu, at radial growth rates between 0.05 and 3.36 (mm day^{-1}). Of the strains that grew in the presence of copper, only 13 grew at rates of 1.60 mm day^{-1} or greater and were selected for the subsequent in vitro tests (Table 2). Despite the high growth rates obtained in the presence of 3 mM Cu, many of the native strains were inhibited by the addition of the metal to the culture medium. The most inhibited native strains (79–80% inhibition) were isolates 7 and 24 of *T. versicolor* and greater inhibitions were observed in *T. versicolor* DEBIQ01 and *G. trabeum* reference strains. By contrast, among the strains most tolerant or least inhibited by the effect of copper (20–30%), *Gloeophyllum* sp. 8, *T. versicolor* 88, and *G. australe* 100 were worthy of note. This last strain was the only one that did not show any significant differences when grown with or without 3 mM Cu (Table 2).

The effect of preservatives on in vitro growth

In order to evaluate the effect of the preservatives on growth in solid medium of the 13 native copper-tolerant strains, a modified test of procedures described by Alleman et al. (1993) was carried out using only one disk impregnated with preservative for each fungal strain, which allowed the effect of the preservatives to be determined as radial growth rate (mm day^{-1}). No diffusion was observed in non-inoculated MEA medium over an 8-day period at the lowest CCA-C concentration. However, a slight

Table 2 Effect of copper on the radial growth rate (mm day^{-1}) of native and reference strains of wood-rot fungi

Strain (N°)	Species	Type of wood-rot	Growth rate (mm day^{-1}) 0 mM Cu	Growth rate (mm day^{-1}) 3 mM Cu	% Inhibition*
7	*Trametes versicolor*	WR	10.60 a	2.15 b	80.00
24	*Trametes versicolor*	WR	11.10 a	2.34 b	79.00
38	*Trametes versicolor*	WR	7.34 a	2.58 b	65.00
73	*Trametes versicolor*	WR	5.41 a	2.09 b	61.00
81	*Trametes versicolor*	WR	7.02 a	1.62 b	77.00
88	*Trametes versicolor*	WR	3.70 a	2.65 b	28.00
28	*Ganoderma australe*	WR	8.80 a	3.36 b	62.00
87	*Ganoderma australe*	WR	4.04 a	1.62 b	60.00
100	*Ganoderma australe*	WR	2.74 a	2.10 a	23.00
119	*Bjerkandera adusta*	WR	3.94 a	1.65 b	58.00
8	*Gloeophyllum* sp.	BR	3.90 a	2.80 b	28.00
132	*Gloeophyllum abietinum*	BR	9.08 a	2.30 b	75.00
139	*Antrodia xantha*	BR	5.82 a	3.23 b	45.00
DEBIQ01	*Trametes versicolor*	WR	6.58 a	1.15 b	83.00
A464	*Ganoderma australe*	WR	6.03 a	2.65 b	56.00
ATCC 62778	*Wolfiporia cocos*	BR	10.00 a	3.16 b	68.00
ATCC 11539	*Gloeophyllum trabeum*	BR	3.56 a	0.71 b	80.00

WR, white-rot; BR, brown-rot

* Growth inhibition due to copper in relation to growth of control without copper

Within a row, growth rate values followed by the same letter have no significant differences ($P < 0.05$)

diffusion around the cellulose disks was observed at the highest CCA-C concentrations and with all BS solutions.

Varied growth responses were obtained between the strains depending on the preservative tested and its concentration. On the other hand, in the same way observed with tolerance to copper, the tolerance to the different concentrations of CCA-C and BS also varied interspecifically as well as intraspecifically. Most strains experienced significant reductions in growth rate when exposed to CCA-C impregnated disks. Some strains, mainly *T. versicolor* (7, 24, 81, and DEBIQ01), *G. australe* 28, and *G. trabeum* had a significant reduction in growth (80–100% inhibition) when exposed to all the concentrations of the CCA-C. By contrast, *G. australe* 87, 100 and A464 and *Gloeophyllum* sp. 8 were highly tolerant to the lowest concentration of CCA-C. Of these, strains 87, 100, and 8 showed no significant growth reduction after the addition of impregnated disks at lowest the CCA-C concentrations tested with respect to the control. Among the reference strains, *W. cocos* was the most tolerant to the highest CCA-C concentration, growing to the edge of the plate in 8 days (Table 3).

The behavior of the different strains in the presence of the different concentrations of BS preservative was as varied as with CCA-C (Table 4). In general, the native strains were not sensitive to the presence of disks impregnated with the BS I solution that contains the lowest concentrations of boron and silicon. In addition, the WRF *T. versicolor* (7 and 38) and *G. australe* (87, 100, and A464), and the BRF *A. xantha* and *Gloeophyllum* sp. (8 and ATCC 11539) showed the least growth reductions of all the BS solutions tested. Among these, *G. australe* 100 and A464 were the only ones that did not show any significant growth reduction compared to the control and *T. versicolor* 38 only experienced a significant growth reduction in the presence of BS IV. Most native and reference strains were highly sensitive to the BS IV solution, which contains the greatest concentrations of boron (0.6%) and silicon (5%) (Table 4). Among the native strains, *T. versicolor* 81 was the most sensitive to BS solutions, mainly to BS II

Table 3 Effect of CCA-C preservative on the radial growth rate (mm day^{-1}) of native and reference strains of wood-rot fungi, using the impregnated disk test

Strain (N°)	Genus/Species	CCA-C concentration (%)			
		0	1	1.5	2.4
7	*Trametes versicolor*	5.39 a	0.96 b	0.66 c	0.59 c
24	*Trametes versicolor*	5.17 a	0.53 b	0.49 b	0.44 b
38	*Trametes versicolor*	6.72 a	3.86 a	4.06 a	3.59 a
73	*Trametes versicolor*	7.15 a	2.99 b	2.57 bc	1.93 c
81	*Trametes versicolor*	6.70 a	0.38 b	0.27 b	0.00 c
88	*Trametes versicolor*	3.52 a	1.62 b	1.82 b	1.56 b
28	*Ganoderma australe*	5.62 a	0.40 b	0.36 b	0.45 b
87	*Ganoderma australe*	2.84 a	2.19 a	1.83 a	0.17 b
100	*Ganoderma australe*	2.93 ab	3.60 a	2.90 ab	2.52 b
119	*Bjerkandera adusta*	4.87 a	1.13 b	1.23 b	0.42 c
8	*Gloeophyllum* sp.	3.26 a	2.70 ab	2.00 bc	1.50 c
132	*Gloeophyllum abietinum*	4.83 a	2.52 b	2.52 b	2.11 b
139	*Antrodia xantha*	5.36 a	3.51 b	2.78 bc	2.08 c
DEBIQ01	*Trametes versicolor*	5.97 a	0.38 b	0.24 c	0.16 c
A464	*Ganoderma australe*	3.58 a	2.65 b	1.01 c	0.94 c
ATCC 62778	*Wolfiporia cocos*	10.87 a	7.13 b	5.60 c	6.00 bc
ATCC 11539	*Gloeophyllum trabeum*	3.19 a	0.53 b	0.37 b	0.20 b

Within a row, growth rate values followed by the same letter have no significant differences ($P < 0.05$)

and IV (89–90% inhibition). On the other hand, *W. cocos* was highly sensitive to all the BS solutions tested and mainly to BS IV (94% inhibition).

Discussion

Most of the fungal native strains isolated in this study grew in the presence of 3 mM Cu in the culture medium, and 13 of them were noteworthy for growth rates greater than 1.6 (mm day^{-1}). Among the strains native to the south-central region of Chile, the *T. versicolor* and *G. australe*, both belonging to the WRF, were the species most frequently found in the ecosystems studied (forest plantations and native forests) in the 2 years of collection (Table 2). On the other hand, in the same collection conditions were found three different isolates belonging to BRF: *Gloeophyllum abietinum*, *Gloeophyllum* sp., and *Antrodia xhanta*. Among the strains that showed the greatest tolerance to 3 mM Cu, reflected through the lowest percentages of inhibition are the strains *T. versicolor* 88, *G. australe* 100, and *Gloeophyllum* sp. 8 (Table 2). Some genera and species identified in this study have been used for years in standard assays for determining the effectiveness of copper-based wood preservatives, such as the BRF *Gloeophyllum* sp. and *A. xantha*, and the WRF *T. versicolor* (Archer et al. 1995; Borokhov and Rothenburger 2000; Pohleven et al. 2002; American Wood Preservers' Association (AWPA) 2004).

When the tolerance to 3 mM Cu was evaluated, a great variety was observed in the growth rate between the different species (interspecific variation) as well as between strains from the same species (intraspecific variation) (Table 2). This behavior was also observed when the effects of CCA-C and BS were evaluated (Tables 3 and 4). This highlights the importance of reporting results based on different strains from one species and of avoiding generalization on the basis of one or only a few strains (Collet 1992; Woodward and De Groot 1999; Clausen et al. 2000). Various studies indicate that in general the BRF present a greater tolerance to Cu and copper-based wood preservatives than WRF, which may be related to the greater production and accumulation of oxalic acid in the BRF cultures (Murphy and Levy 1983; Baldrian 2003; Green and Clausen 2005);

Table 4 Effect of BS preservative on the radial growth rate (mm day^{-1}) of native and reference strains of wood-rot fungi, using the impregnated disk test

Strain (N°)	Genus/Species	BS solution				
		0	I	II	III	IV
7	*Trametes versicolor*	5.39 a	3.63 b	4.63 ab	3.99 b	3.97 b
24	*Trametes versicolor*	5.17 a	4.17 a	2.88 b	2.72 b	1.93 b
38	*Trametes versicolor*	6.72 a	4.93 ab	4.75 ab	4.56 ab	3.71 b
73	*Trametes versicolor*	7.15 a	5.47 b	4.73 b	5.03 b	2.97 c
81	*Trametes versicolor*	6.70 a	4.09 b	0.67 d	2.72 c	0.76 d
88	*Trametes versicolor*	3.52 a	2.41 b	2.27 b	1.71 c	1.39 c
28	*Ganoderma australe*	5.62 a	4.02 b	2.31 c	2.57 c	1.67 c
87	*Ganoderma australe*	2.84 a	3.27 a	2.30 b	2.09 bc	1.53 c
100	*Ganoderma australe*	2.93 ab	3.89 ab	4.47 a	3.67 ab	3.20 b
119	*Bjerkandera adusta*	4.87 a	4.44 ab	2.62 c	3.39 bc	2.09 c
8	*Gloeophyllum* sp.	3.26 a	2.22 bc	2.40 b	2.50 b	1.72 c
132	*Gloeophyllum abietinum*	4.83 a	3.52 b	2.74 c	1.81 d	2.47 cd
139	*Antrodia xantha*	5.36 a	5.30 a	4.28 b	4.76 ab	4.26 b
DEBIQ01	*Trametes versicolor*	5.97 a	4.06 b	2.76 c	2.55 c	2.62 c
A464	*Ganoderma australe*	3.58 a	3.30 a	2.55 a	2.36 a	2.25 a
ATCC 62778	*Wolfiporia cocos*	10.87 a	4.90 bc	5.20 b	4.13 c	0.65 d
ATCC 11539	*Gloeophyllum trabeum*	3.19 a	3.43 a	2.43 b	2.27 b	1.80 b

Within a row, growth rate values followed by the same letter have no significant differences ($P < 0.05$)

however, most studies have made comparisons using a single strain for each species analyzed and few investigations include different strains from the same species, be it WRF or BRF. *T. versicolor*, from the WRF group, has been described as a copper-sensitive species (Pohleven et al. 2002; Humar and Pohleven 2005); nevertheless, our results show that despite the majority of native strains of *T. versicolor* being extremely sensitive to Cu, one of them stood out for its heightened tolerance to the metal (strain 88). Moreover, due to the low amount of BRF collected, it was only possible to assay three different strains, of which one belonging the genus *Gloeophyllum* exhibited elevated tolerance (*Gloeophyllum* sp. 8).

The test using disks impregnated in preservatives enabled native strains of wood-rot fungi sensitive and/or tolerant to CCA-C, BS, or both to be identified. Most of the native or reference strains presented greater sensitivity to the presence of CCA-C than BS in the concentrations tested. The greatest levels of tolerance to CCA-C were obtained with the WRF *T. versicolor* 38 and *G. australe* 100 and with the BRF *Gloeophyllum* sp. 8 and *W. cocos*, of these, strains 100 and 8 also exhibited a high tolerance to 3 mM Cu. By contrast, some of the strains that showed a high inhibition to 3 mM Cu also presented a strong and significant reduction in growth in the presence of CCA-C. These results suggest that there may be a correlation between the effect of copper on the growth of some strains in solid medium and their growth in the presence of CCA-C, confirming that the plate tests for copper tolerance are useful for selecting new strains tolerant to the traditional preservatives that contain copper. Some of the strains tolerant to CCA-C also showed a great tolerance to non copper-based BS preservative, in particular *G. australe* 100, whose growth was not inhibited by any of the BS solutions tested. Also, the *A. xantha*, belonging to a genus known in the literature for its copper tolerance (Collet 1992; Illman and Highley 1996; Illman et al. 2000), showed higher tolerance to BS preservative than CCA-C. On the other hand, the recognized copper-based preservative tolerant fungus, *W. cocos*, showed a greater sensitivity to BS preservative, mainly to the BS IV solution.

Boron acts as a fungicide and insecticide has been used for years in wood preservatives alone or with copper (CCB) or with silicon, as in the case of the BS preservative, and the efficiency of boron has been evaluated against different wood-rot fungi. The boron-based preservatives are generally not as hard on the environment as copper-based preservatives (Lebow 2004; Przewloka 2004; Humar et al. 2007). Due to the newness of the BS solution, a preservative only in use recently in the preserved wood market in Chile (Navarrete 2004), these are the first results described in the literature that show the effect of this new formulation on growth in solid medium of a variety of native and commercial strains of wood-rot fungi, using the impregnated disk test. This test also enabled the effect of this non copper-based preservative to be compared with the well-known and widely used commercial copper-based preservative (CCA-C) with respect to the growth of the different strains of wood-rot fungi.

The *Ganoderma* genus has been reported for its tolerance to metals in the culture medium, among them copper (Tham et al. 1999; Baldrian 2003; Guillén and Machuca 2008); to our knowledge, however, there is no information in the literature that mentions the behavior of *G. australe* with respect to copper-based or boron–silicon-based wood preservatives, be it in in vitro tests, as conducted here, or in tests using preserved wood. However, there are data that reveal the ability of *G. australe* A464 to degrade 2,4,6-tribromophenol (TBP) in liquid culture medium, a much greater degradation than that produced by the BRF *G. trabeum* and *Laetiporus sulfureus* (Monrroy et al. 2006, 2007). The native strain *G. australe* 100 was remarkable in all the tests performed due to its great tolerance to 3 mM Cu in solid medium and to its tolerance to the different concentrations of CCA-C and BS in tests using impregnated disks. This strain turned out to be of great interest for future studies related to the mechanisms involved in the tolerance to heavy metals and wood preservatives, and, alternatively, in assays of biodegradation of waste preserved wood. Currently under evaluation is the potential of the native strains isolated in the south-central Chile that showed the greatest tolerance to both preservatives in order to colonize and degrade *Pinus radiata* wood impregnated with CCA-C and BS preservatives.

Acknowledgments The authors wish to thank the valuable technical support of Forest Engineer Daniel Chávez M. We also wish to thank A. Franzani and O. Inostroza for donating the CCA-C and BS preservatives.

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