

# The effect of $\text{CaCl}_2$ on growth rate, wood decay and oxalic acid accumulation in *Serpula lacrymans* and related brown-rot fungi

Anne Christine Steenkjær Hastrup<sup>1,\*</sup>, Bo Jensen<sup>1</sup>, Carol Clausen<sup>2</sup> and Frederick Green III<sup>2</sup>

<sup>1</sup> Department of Microbiology, Biological Institute, University of Copenhagen, Copenhagen, Denmark

<sup>2</sup> US Department of Agriculture Forest Service, Forest Products Laboratory, Madison, WI, USA

\*Corresponding author.

University of Copenhagen, Institute of Biology, Department of Microbiology, Sølvgade 83H, 1307 Copenhagen K, Denmark

Tel.: +45-20-235937

Fax: +45-35-322321

E-mail: anneh@bi.ku.dk

## Abstract

The dry rot fungus, *Serpula lacrymans*, is one of the most destructive copper-tolerant fungi causing timber decay in buildings in temperate regions. Calcium and oxalic acid have been shown to play important roles in the mechanism of wood decay. The effect of calcium on growth and decay was evaluated for 12 strains of *S. lacrymans* and compared to five brown-rot fungi. This was done by treating copper citrate (CC)-treated Southern yellow pine (SYP) wood with a  $\text{CaCl}_2$  solution and estimating the decay rate and amount of soluble oxalic acid in an ASTM soil block test. Decay by *S. lacrymans* was found to be significantly inhibited by treatment with  $\text{CaCl}_2$  in the presence of copper. In addition, calcium showed no effect on two strains of *S. lacrymans* and one *Serpula himantioides* strain in non-copper-treated SYP wood blocks. The growth rate of *S. lacrymans* was not affected on malt extract agar containing  $\text{CaCl}_2$ . In summary, a marked decrease was observed in the decay capacity of *S. lacrymans* in pine treated with CC+ $\text{CaCl}_2$ . The amount of soluble oxalic acid was measured in CC-treated blocks and blocks also treated with  $\text{CaCl}_2$ . Of the comparative brown-rot fungi, both *Antrodia vaillantii* (TFFH 294) and *Postia placenta* (Mad 698) displayed notable wood decay despite  $\text{CaCl}_2$  treatment, while the remaining strains were inhibited.

**Keywords:** brown-rot fungi; calcium;  $\text{CaCl}_2$ ; copper citrate (CC); copper-based preservative; HPLC; oxalic acid; *Serpula lacrymans*; soil bottle test; wood decay.

## Introduction

The dry rot fungus *Serpula lacrymans* (Wulfen:Fries) Schröter (1889) is one of the most destructive and impor-

tant decay fungi in buildings in Northern and Central Europe. It holds special fascination and notoriety as a unique wood-rotting fungus owing to its ability to cause decay and structural damage in both timber and masonry. The rare appearance of the dry rot fungus in houses built entirely from wood and its apparent relatively sparse colonisation of forest wood and litter led to the theory that the fungus requires a calcium source, i.e., alkaline building materials, to survive the accumulation of oxalic acid that takes place during wood decay (Bech-Andersen 1987b, 1989, 2004). However, high oxalic acid accumulation by other brown-rot fungi, e.g., *Tyromyces palustris* (Clausen and Green 2003), is not addressed, since these fungi are not found in connection with alkaline building materials.

Oxalic acid ( $\text{C}_2\text{H}_2\text{O}_4$ ; OA) is a small organic acid ( $\text{p}K_1=1.27$ ,  $\text{p}K_2=4.26$ ) assumed to be a metabolic by-product of incomplete glucose oxidation either via malate in the TCA cycle or glyoxylate in the GLOX cycle (Gadd 1999; Munir et al. 2001). Synthesis of OA by brown- and white-rot fungi was first documented by Schimazono and Takubo (1952). It is known to be produced in measurable amounts by the majority of brown-rot fungi, including *S. lacrymans*, but only in limited amounts by white-rot fungi because of the presence of the OA-degrading enzyme oxalate decarboxylase (ODC) (Akamatsu et al. 1992). OA has been ascribed many roles in brown- and white-rot decay (Takao 1965; Green and Clausen 2003). In brown-rot fungi it is attributed a rapid lowering of pH in the immediate environment (Shimada et al. 1994), the initiation of hemicellulose side-chain breakage and hemicellulose depolymerisation (Green et al. 1991), and precipitation of calcium as calcium oxalate during decomposition to avoid toxic levels. Calcium, being the most abundant metal ion found in wood, is released during decay (Whitney and Arnott 1987; Sayer and Gadd 1997) and translocation of calcium is argued to function in pH regulation by precipitating OA to form the nearly water-insoluble calcium oxalate (Connolly et al. 1996).

It has been proposed that OA accumulation without the presence of a cation buffer such as calcium creates a problem for the survival of *S. lacrymans* (Bech-Andersen 1989). To test this assumption, the effect of a calcium source,  $\text{CaCl}_2$ , was examined with regard to accumulation of soluble OA. This compound was chosen because no effect was found on the growth rate of *S. lacrymans* as a result of  $\text{CaCl}_2$  treatment (Palfreyman et al. 1996).

The objective of this study was to investigate the influence of calcium ions on the decay rate of copper- and non-copper-treated wood, on the growth rate of *S. lacrymans* and comparative brown-rot fungi using  $\text{CaCl}_2$  solution, and the effects of OA accumulation in *S. lacrymans*.

**Table 1** *Serpula lacrymans* strains used in the study.

| Strain              | Date of isolation | Original coding/designation | Origin              |
|---------------------|-------------------|-----------------------------|---------------------|
| Bb 29 <sup>1</sup>  | 1988              | MUCL30055                   | Belgium, Baisy-Thy  |
| SI 199 <sup>2</sup> | 1978              | S22/HFP7802                 | Japan, Asahikawa    |
| SI 200 <sup>2</sup> | 1953              | S27                         | Poland, Warsaw      |
| SI 202 <sup>2</sup> | 1965              | S29/BF1                     | France, Xylochimie  |
| SI 207 <sup>2</sup> | 1971              | S32                         | England, Rothsay    |
| SI 209 <sup>2</sup> | 1984              | S37                         | Germany, Velbert    |
| SI 216 <sup>2</sup> | 2003              |                             | Norway, Fannrem     |
| SI 217 <sup>2</sup> | 2003              |                             | Norway, Oslo        |
| SI 219 <sup>2</sup> | 1955              | 311                         | Finland, Helsinki   |
| SI 221 <sup>2</sup> | 1947              | 6                           | Finland, Helsinki   |
| Bam Ebers 315       | 1936              | EMPA 65                     | Germany, Eberswalde |
| ATCC 11485          | 1946              | FP-90876-R                  | USA, Asheville, NC  |

<sup>1</sup>Identification code used by Technological Institute, Denmark.

<sup>2</sup>Identification code used by Håvard Kauserud, Biological Institute, Oslo University, Norway.

## Materials and methods

### Fungal cultures

A total of 12 *S. lacrymans* strains from the northern hemisphere (Table 1) were used for individual comparisons and evaluation of species variation. Five brown-rot fungi were included for comparison: *S. himantoides* (ATCC 36335, Sh 100), *Antrodia vaillantii* (TFFH 294), *Tyromyces palustris* (TYP 6137), *Gloeophyllum trabeum* (Mad 617), and *Postia placenta* (Mad 698). All fungi were maintained on 2% malt extract agar (Difco Laboratories, Detroit, MI, USA).

The two strains ATCC 36335 and TFFH-294 were re-identified. The internal transcribed spacer (ITS) region was extracted (Murray and Thompson 1980) using primers ITS5 and ITS4 (Schmidt 2000), and a BLAST search in GenBank (www.ncbi.nlm.nih.gov) showed that ATCC 36335 is *Serpula himantoides* (GenBank accession no. AM076558), while TFFH-294 aligned with sequences from *Antrodia vaillantii* (GenBank accession no. DQ121432).

### Effect of calcium on the decay of copper-treated wood

Southern yellow pine (SYP) sapwood blocks (10 mm×10 mm×10 mm) and SYP sapwood feeder strips (3 mm×28 mm×35 mm) were conditioned to 12.6% equilibrium moisture content and weighed. Blocks were pressure-treated with 1.2% ammoniacal copper citrate (CC) to the American Wood Preservers' Association (AWPA) standard retention. Weighed blocks were submerged in solution and subjected to a vacuum of -165.5 kPa gage pressure twice for 20 min each. Blocks were weighed, dried in a fume cupboard overnight, returned to the conditioning room for 1 week, and reweighed.

Dried pressure-treated wood block samples were ground to pass a US standard 20-mesh (850 µm) screen, digested, and analysed for copper and calcium retention by ICP according to AWPA standard A21-00. The retention for CC-treated blocks was 4.6 mg g<sup>-1</sup> Cu. Half of the CC-treated blocks were also pressure-treated with a 1% (w v<sup>-1</sup>) CaCl<sub>2</sub>-solution, with retention of 5.8 mg g<sup>-1</sup> Cu and 3.2 mg g<sup>-1</sup> Ca. Untreated blocks served as controls. The wood blocks were placed on pre-inoculated SYP wood block feeders (3 mm×28 mm×35 mm, with the small dimension in the fibre direction) on topsoil in standard square ASTM culture bottles (450 ml). Blocks were subjected to the test fungi in a soil-block test (ASTM standard D143-76) following the guidelines in AWPA standard E10-01. Soil bottles were incubated at 70% relative humidity (RH) for 10 weeks at 20°C (*S. lacrymans*) or 27°C (remaining five fungi species), according to the

recommended growth temperature. Five replicates of treated and untreated blocks for each fungus were tested after incubation for 1, 2, 4, 6, 8, and 10 weeks. Following incubation, blocks were removed from bottles, brushed free of mycelium, weighed, oven-dried at 60°C for 24 h, weighed, conditioned to 70% equilibrium moisture content, and re-weighed. Percentage weight loss was calculated from the treated conditioned weights before and after decay testing.

### Effect of calcium on the decay of non-copper treated wood

The effect of calcium on the decay capacity of fungal strains in non-copper-treated wood was monitored following the guidelines for the soil block test described above with some modifications. *S. lacrymans* SI 202 and SI 221 were chosen, since they represented the mean and highest values for decay capacity, respectively, for the 12 strains of *S. lacrymans* in soil block test with copper-treated wood. *S. himantoides* ATCC 36335 was also tested, since it was previously regarded as *S. lacrymans*, and variation between the two species was compared. Pressure-treated wood blocks and feeder strips were treated with a 1% (w v<sup>-1</sup>) CaCl<sub>2</sub> solution, with retention of 2.6 mg g<sup>-1</sup> Ca. Untreated blocks served as controls.

### Toxicity tests

The effect of calcium on the growth rate of two *S. lacrymans* strains, SI 209 and SI 219, and the effect when incorporated with CC was determined by including CaCl<sub>2</sub> at concentrations of 0.001%, 0.01%, and 0.1% w v<sup>-1</sup> in 1.5% malt extract agar (MEA) plates, with and without CC at the corresponding concentrations (0.001%, 0.01%, and 0.1% w v<sup>-1</sup>, respectively). Fungal strains were chosen on the basis of their performance in the soil block test, representing strains with high and little copper tolerance, respectively (Hastrup et al. 2005b). Media pH was standardised to 4.5 after addition of the alkaline solution and before sterilisation. The pH of 1% aqueous CC and CC+CaCl<sub>2</sub> solutions was 9.7. Plates were incubated for 2 weeks at 20°C and radial growth was compared in MEA plated with CC, CC+CaCl<sub>2</sub> and control 1.5% MEA.

### Determination of OA

Each block was extracted in 3.0 ml of 0.1 M phosphate buffer, pH 7.0 (Green and Clausen 2003) for 24 h while mixing on a rotation platform at 100 rpm. Extraction solutions were subjected to HPLC on a LiChroCart® Purospher® STAR RP-18 column (particle size 5 µm, 250×4.6 mm i.d.) for OA analysis (Merck &

Co., Inc., Whitehouse Station, NJ, USA) on an Äkta Purifier A-900 HPLC model with a P-900 autosampler (Amersham Pharmacia Biotech, Buckinghamshire, England). The retention time of OA was 3.10 min.

OA was chromatographed to determine the efficiency of the column, optimal wavelength for reading, retention time, and to produce a standard curve. Extraction samples were centrifuged for 3 min at 13 000 rpm, and 25- $\mu$ l aliquots of the sample solutions were autoinjected into the chromatographic system. The mobile phase used was 25 mM phosphate buffer, pH 2.5, with detection at 210 nm to avoid monitoring of proteins, enzymes, etc. The run time was 14 min to allow for detection of other potential acids. Quantification was based on peak area.

Verification of OA peaks was done by spiking with known amounts of OA (100  $\mu$ l, 5 mM) (Sayer and Gadd 1997) or by adding 50  $\mu$ l of 0.5 M CaCl<sub>2</sub> and spinning the solution for 3 min at 13 000 rpm to precipitate soluble OA. Controls were prepared for each solution and for uninoculated blocks treated with each solution.

## Results

### Effect of CaCl<sub>2</sub> on the decay of copper-treated wood

The effect of calcium was evaluated by comparing the decay capacity of fungi grown on copper-treated blocks with and without calcium treatment (Table 2). CC-treated wood blocks also treated with CaCl<sub>2</sub> showed remarkable resistance to decay for all fungal strains, including *S. lacrymans*. Only *Antrodia vaillantii* TFFH-294 and *P. placenta* Mad 698 showed significant decay, with an average weight loss > 10% (t-test,  $P < 0.05$ ). Results for *S. lacrymans* isolates displayed an average weight loss of only 3% for CC + CaCl<sub>2</sub>-treated wood blocks. Only one isolate, SI 209, differed from the mean, showing a slight

weight loss in these blocks (~8%). The average weight losses were 31% for CC-treated wood blocks without CaCl<sub>2</sub> and 40% for the control wood blocks. For Bb 29, CC treatment of wood seemed to improve the decay capacity of the fungal strain compared to the control; however, the increase was not statically significant (t-test,  $P > 0.05$ ).

### Effect of calcium on the decay of non-copper-treated wood

The effect of calcium in non-copper-treated blocks on the decay rate of *S. lacrymans* and *S. himantioides* using soil bottle tests did not show any notable differences in weight loss among CaCl<sub>2</sub>-treated strips, CaCl<sub>2</sub>-treated wood blocks, and control blocks. The average weight loss for wood blocks inoculated with SI 202 and SI 221 ranged from 40% in CaCl<sub>2</sub>-treated wood blocks to 47% when grown on CaCl<sub>2</sub>-treated feeder strips. In neither of the treatment groups did the average weight loss significantly exceed the results for control blocks (one-way ANOVA, means comparison test for three groups,  $P > 0.3$ ). In addition, *S. himantioides* ATCC 36335 showed a lower average weight loss than *S. lacrymans* for all treatments, although the result was not statistically significant (one-way ANOVA,  $P > 0.05$ ).

### Toxicity tests

Growth inhibition of *S. lacrymans* strains SI 209 and SI 219 on 2% MEA plates by three concentrations of CC and CC + CaCl<sub>2</sub> are shown in Table 3. The highest concentration (0.1%) of CC and CC + CaCl<sub>2</sub> was not enough to completely inhibit growth of the two strains, and therefore no difference was observed between plates with CC

**Table 2** Weight losses in wood blocks treated with copper citrate (CC) and CC + CaCl<sub>2</sub> compared to controls in soil bottle tests for 10 weeks (n = 5).

| Fungal strain          | Mean weight loss (%) |                        |                 |
|------------------------|----------------------|------------------------|-----------------|
|                        | CC                   | CC + CaCl <sub>2</sub> | Control         |
| <i>S. lacrymans</i>    |                      |                        |                 |
| SI 221                 | 46.7 $\pm$ 5.3       | 3 $\pm$ 0.3            | 46.2 $\pm$ 5.2  |
| Bb 29                  | 38.3 $\pm$ 5.8       | 2 $\pm$ 0.4            | 31.5 $\pm$ 13.7 |
| ATCC 11485             | 33.1 $\pm$ 10.8      | 3.9 $\pm$ 0.1          | 38.1 $\pm$ 7.1  |
| SI 219                 | 32.9 $\pm$ 18.7      | 2.1 $\pm$ 0.3          | 53.1 $\pm$ 6.9  |
| SI 216                 | 32.3 $\pm$ 14.0      | 2.3 $\pm$ 0.3          | 44.6 $\pm$ 12.5 |
| SI 202                 | 31.6 $\pm$ 3.2       | 3 $\pm$ 0.1            | 44.0 $\pm$ 10.6 |
| SI 200                 | 29.3 $\pm$ 11.5      | 2.0 $\pm$ 0.1          | 31.4 $\pm$ 9.2  |
| SI 217                 | 29.0 $\pm$ 9.9       | 1.9 $\pm$ 0.8          | 42.2 $\pm$ 5.5  |
| SI 207                 | 29.0 $\pm$ 7.9       | 3 $\pm$ 0.6            | 33.1 $\pm$ 8.6  |
| SI 199                 | 27.7 $\pm$ 6.5       | 2 $\pm$ 0.5            | 27.5 $\pm$ 4.2  |
| SI 209                 | 27.4 $\pm$ 11.6      | 8.1 $\pm$ 6.2          | 38.7 $\pm$ 5.8  |
| Bam Ebers 315          | 9.8 $\pm$ 6.5        | 3.3 $\pm$ 0.5          | 45.0 $\pm$ 9.0  |
| <i>S. himantioides</i> |                      |                        |                 |
| Sh 100                 | 45.9 $\pm$ 10.5      | 1.4 $\pm$ 0.6          | 47.7 $\pm$ 3.8  |
| ATCC 36335             | 28.4 $\pm$ 15.3      | 2.0 $\pm$ 0.1          | 38.7 $\pm$ 4.5  |
| <i>P. placenta</i>     |                      |                        |                 |
| Mad 698                | 57.0 $\pm$ 11.5      | 13.5 $\pm$ 4.0         | 68.5 $\pm$ 1.9  |
| <i>T. palustris</i>    |                      |                        |                 |
| TYP 6137               | 39.6 $\pm$ 9.4       | 3.1 $\pm$ 17.4         | 48.2 $\pm$ 6.4  |
| <i>G. trabeum</i>      |                      |                        |                 |
| Mad 617                | 4.8 $\pm$ 7.2        | 0.1 $\pm$ 0.5          | 59.4 $\pm$ 8.0  |
| <i>A. vaillantii</i>   |                      |                        |                 |
| TFFH-294               | 20.0 $\pm$ 5         | 12.0 $\pm$ 0.1         | 11.6 $\pm$ 4    |

**Table 3** Growth reduction of two strains of *S. lacrymans* tested on CC- and CaCl<sub>2</sub>-containing MEA plates.

| Strain | Growth change (%) |       |      |                        |       |      |                   |       |      |
|--------|-------------------|-------|------|------------------------|-------|------|-------------------|-------|------|
|        | CC                |       |      | CC + CaCl <sub>2</sub> |       |      | CaCl <sub>2</sub> |       |      |
|        | 0.001%            | 0.01% | 0.1% | 0.001%                 | 0.01% | 0.1% | 0.001%            | 0.01% | 0.1% |
| SI 209 | 0                 | 0     | -32  | 0                      | 0     | 0    | -27               | -4    | -9   |
| SI 219 | -26               | -13   | -42  | -13                    | -18   | -31  | -11               | 4     | 28   |

Growth reduction is expressed as increased or decreased radial growth in test plates compared to the control (n=3).

and with CC + CaCl<sub>2</sub>. Plates with CaCl<sub>2</sub> alone seemed to induce growth in strain SI 219 (Table 3).

### HPLC measurements of soluble OA

HPLC was used to assay extracts from blocks inoculated with five *S. lacrymans* strains representing low, moderate, and high copper tolerance (Bam Ebers 315; SI 209, SI 219, and SI 221; and Bb29, respectively) for soluble OA accumulated over 10 weeks of incubation. Figure 1 shows a higher amount of soluble OA in copper-treated blocks compared to untreated blocks. The highest amount of soluble OA was measured in CC-treated blocks. Control blocks had the lowest amount of soluble OA for all strains. Blocks inoculated with Bam Ebers 315 showed the lowest amounts of measurable OA among the five *S. lacrymans* strains. The OA concentration and the percentage decay increased in blocks for all strains from week 1 to week 6, with a decrease in some block groups after 8 weeks, which was followed either by an increase at week 10 or a levelling off. Only one strain, SI 219, did not show an increase in OA concentration by week 10.

Analysis of the OA measured as a function of treatment showed that CaCl<sub>2</sub> affected the amount of soluble OA accumulated and the decay rate (Figures 1 and 2). Untreated control blocks showed a high decay rate with relatively low OA accumulation compared to blocks treated with CC, which showed a linear correlation between OA production and weight loss. In both these groups,

the OA levels declined when the weight loss reached 45–50%. Wood blocks inoculated with SI 219 that were degraded by more than 50% also showed a decreasing amount of soluble OA (Figure 2).

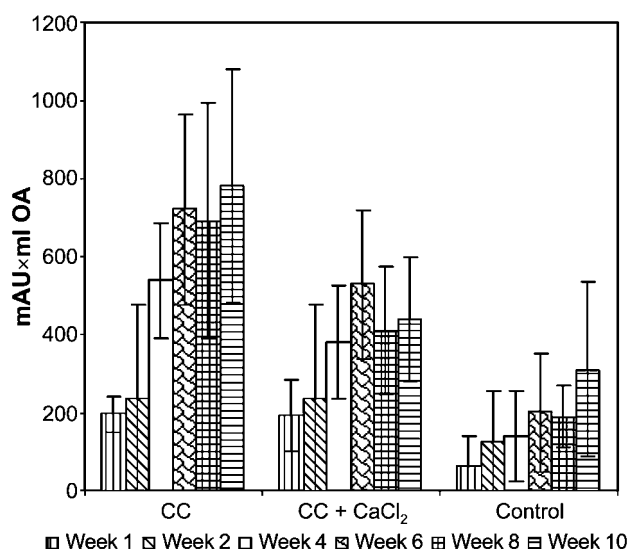
Figure 3 shows that the average amount of soluble OA accumulated in *T. palustris* was higher than the average result for *S. lacrymans* during all the weeks and for all solutions.

## Discussion and conclusion

### Effect of calcium

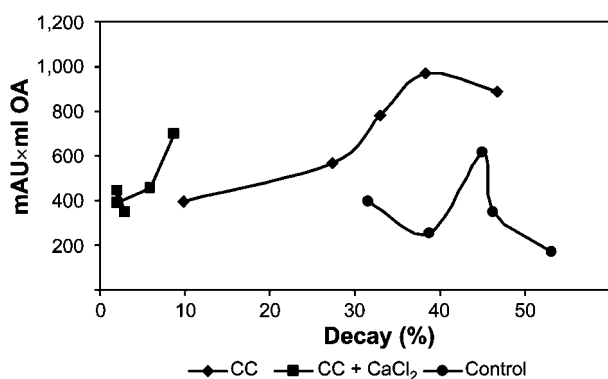
Decay in CC + CaCl<sub>2</sub>-treated wood blocks inoculated with *S. lacrymans* (Table 2) was insignificant for 11 of the 12 strains (t-test,  $P < 0.05$ ) within experimental error, as the weight loss was  $< 5\%$  (Clausen and Kartal 2003). *S. himantoides* (ATCC 36335, Sh 100), *T. palustris* (TYP 6137) and *G. trabeum* (Mad 617) were similarly inhibited and caused no decay. However, *A. vaillantii* TFFH-294 and *P. placenta* Mad 698 caused significant decay (t-test,  $P < 0.05$ ). This partial tolerance to CC + CaCl<sub>2</sub>-treated wood observed for TFFH-294 and Mad 698 was correlated with a high tolerance to copper. Despite the high standard deviation of the results for Mad 698, the high weight losses observed in some blocks indicate that this strain is capable of withstanding CaCl<sub>2</sub> treatment.

Results for the CC-containing agar plate tests showed that CaCl<sub>2</sub> did not affect the growth rate of the *S. lacry-*



**Figure 1** Accumulation of oxalic acid in SYP wood blocks treated with copper citrate, copper citrate + CaCl<sub>2</sub>, or untreated, subjected to soil bottle tests for 1, 2, 4, 6, 8, or 10 weeks. The results presented are the average oxalic acid measured by HPLC for *S. lacrymans* strains Bb 29, Bam Ebers 315, SI 209, SI 219, and SI 221. Bars represent the standard deviation (n=30).





**Figure 2** Correlation between weight loss (% decay) and accumulated soluble oxalic acid for copper citrate (CC)-treated, CC +  $\text{CaCl}_2$ -treated, and untreated wood blocks incubated for 10 weeks in a soil bottle test with five *S. lacrymans* strains (Bam Ebers 315, Bb 29, SI 209, SI 219 and SI 221). Each plot represents the average of results measured for five replicate blocks for each of the strains.

*mans* strains except at high concentrations. These results indicate, as previously reported, that calcium and chloride may affect the decay process, but not the growth rate (Palfreyman et al. 1996). A probable explanation for the influence on decay and not growth rate is the precipitation of OA as calcium oxalate, which may lower the amount of OA available or increase the already acidic conditions produced by *S. lacrymans*. When a calcium ion from  $\text{CaCl}_2$  is precipitated by OA, it is replaced by a proton ( $\text{H}^+$ ), making the environment more acidic (Palfreyman et al. 1996). These explanations indicate that calcium chloride cannot be used as a  $\text{Ca}^{2+}$  source for evaluation of the effect of calcium on decay by *S. lacrymans*. However, the compound can reveal other interesting aspect, e.g., for use in wood preservation.

The fungal inhibitory effect of  $\text{CaCl}_2$  treatment observed in CC-treated blocks (Table 2) was not ob-

served in non-copper-treated blocks, for which weight loss was the same as that of the control blocks. These results and the agar plate tests, which did not show a tendency toward growth inhibition (Table 2), indicate that the effect of  $\text{CaCl}_2$  is not directly fungicidal, in accordance with Palfreyman et al. (1996). It is proposed that the effect of  $\text{CaCl}_2$  is the result of a synergistic effect of the reaction between CC and  $\text{CaCl}_2$ , with  $\text{Ca}^{2+}$  functioning as a sink for OA and resulting in the retention of free copper ions in the wood blocks (Figure 2). Only a salt concentration of 1% w v<sup>-1</sup> was used, since at higher concentrations the salt and not calcium oxalate could possibly be a factor in limiting decay.

*S. lacrymans* is known to be copper-tolerant (Hastrup et al. 2005a,b); therefore, in blocks treated solely with CC, the wood is not protected by the preservative and the decay observed is the result of remaining OA, despite calcium oxalate precipitation, or the effect of other possible mechanisms involved in dry rot decay (Green and Highley 1997).

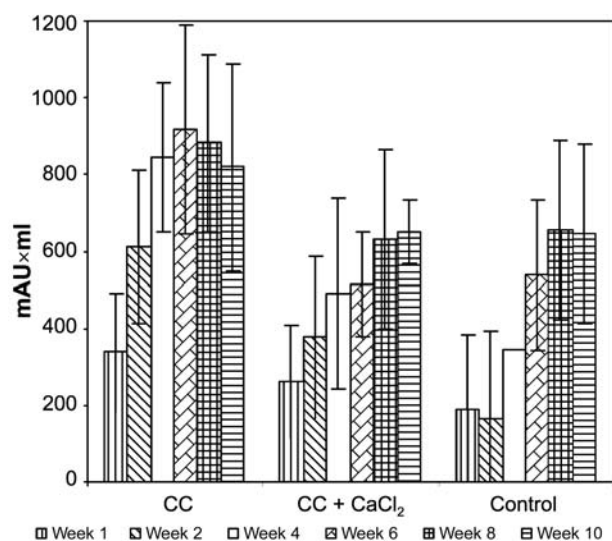
In summary,  $\text{CaCl}_2$  functions as an OA sink, leaving Cu ions in solution to act as a fungicide.

### OA and wood decay

Figure 2 shows correlation between the amount of soluble OA and the degree of wood decay in untreated control blocks, which indicates that OA levels decline in heavily degraded wood. In this case, such a phenomenon occurred at a weight reduction of ~45–50%. This decrease is in agreement with reports that the amount of soluble OA were almost undetectable in wood blocks that were highly to completely degraded (Espejo and Agosin 1991; Itakura et al. 1994). These authors also found that the amount of OA was inversely correlated with the degradation activity of fungi against wood, crystalline cellulose, and hydroxyl radical production.

The CC +  $\text{CaCl}_2$ -treated wood blocks generally showed a lower amount of soluble OA compared to the CC-treated wood blocks, which can be explained by the extra OA sink present, i.e., calcium (Dutton and Evans 1996; Humar et al. 2002), or by overall lower production of OA. An acidified extraction solution could have been used to dissolve calcium oxalate or copper oxalate for HPLC analysis, which would have given a picture of the total OA production (Schilling and Jellison 2004), since calcium oxalate is soluble below pH 5.0 (Gadd 1999). The presence of OA in wood blocks treated with CC +  $\text{CaCl}_2$  indicates an indirect fungicidal effect and most likely reflects inhibition of the decay process in other ways.

The hypothesis that *S. lacrymans* depends on calcium to neutralise OA and thus prevent the growth media from becoming exceedingly acidic appears attractive (Bech-Andersen 1987a); however, this has not been suggested for other brown-rot fungi. *T. palustris* was found to produce higher quantities of soluble OA than the most copper-resistant *S. lacrymans* strain, Bb 29 (Figure 2 and Figure 3), with correspondingly higher decay in CC-treated and untreated wood blocks (Table 2). In contrast to the *S. lacrymans* strains, the addition of calcium inhibited the decay capacity of *T. palustris*. Tests for other non-calcium-dependent brown-rot basidiomycete support these results (Green et al. 1991). It has been speculated



**Figure 3** Accumulation of soluble oxalic acid in SYP wood blocks treated with copper citrate, copper citrate and  $\text{CaCl}_2$ , or untreated, subjected to soil bottle tests for 1, 2, 4, 6, 8, or 10 weeks, and inoculated with *Tyromyces palustris* TYP 6137. Oxalic acid was measured by HPLC. Bars represent the standard deviation (n=5).

that a switch-off mechanism to regulate OA production perhaps did not develop in *S. lacrymans* because of a certain metal ion composition of the soil in the habitat of origin (Palfreyman et al. 1995). However, the possibility that *S. lacrymans* requires an external buffering system for controlling OA levels is considered unlikely (Paajanen and Ritschkoff 1991). The mechanisms behind OA tolerance that presumably separate *T. palustris* and other brown-rot fungi from *S. lacrymans* are still to be discovered.

### Copper tolerance versus OA production

The HPLC results for extracts of *S. lacrymans*-inoculated wood blocks showed induction of soluble OA production by CC treatment. Blocks treated with CC showed an average concentration of soluble OA ranging from 400 to ~1000 mAU×ml by week 10, while the concentration in the control group did not exceed 600 mAU×ml (Figure 1). A similar consistently higher level of soluble OA in copper-treated blocks by week 10 was observed by Green and Clausen (2001), regardless of the precipitation of insoluble calcium oxalate and copper oxalate. These results point to the increased amount of soluble OA in the copper-treated blocks as a factor that diminishes the toxic effect of copper, i.e., because of lower availability of Cu ions in the wood. The end result is a decay rate similar to that for untreated wood. This is in agreement with the proposed mechanism for OA in regard to copper tolerance and the ability of some wood decay fungi to immobilise copper by precipitating copper oxalate (Sutter et al. 1983; Woodward and De Groot 1999). A lower accumulation of soluble OA was observed in Bam Ebers 315 compared to the other *S. lacrymans* strains (data not shown), as was also observed by Green and Clausen (2003). This correlates with the intolerance toward copper that was registered for this strain and adds further support to the assumption that OA is highly involved in copper tolerance. The results from the present study strongly support the hypothesis that OA is involved in copper tolerance.

In summary, CaCl<sub>2</sub> functions as an OA sink, leaving Cu ions in the solution to act as a fungicide. The inhibitory effect of CaCl<sub>2</sub> observed in copper-treated wood and not in untreated wood, along with the fact that growth was not inhibited by CaCl<sub>2</sub>, indicates that the compound itself is not inhibitory.

### Acknowledgements

The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service. The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright. The authors wish to thank Rachel Arango, FPL, for her technical assistance, Håvard Kauserud, Oslo University, Norway, for providing cultures and assistance with molecular tests, Morten Klammer, Technological Institute, Denmark, for providing cultures, and Jørgen Bech-Andersen for interesting discussions.

### References

- Akamatsu, Y., Takahashi, M., Shimada, M. (1992) Cell-free extraction and assay of oxaloacetate from the brown-rot fungus *Tyromyces palustris*. *Mokuzai Gakkaishi* 38:495–500.
- ASTM standard (1998) D143-76. Test method for wood preservatives by laboratory soil-block cultures.
- AWPA standard (2001) A21-00. Standard method for the analysis of wood and wood treating solutions by inductively coupled plasma emission spectrometry.
- AWPA standard (2003) E10-01. Standard method of testing wood preservatives by laboratory soil-block cultures.
- Bech-Andersen, J. (1987a) Production, function and neutralization of oxalic acid produced by the dry rot fungus and other brown rot fungi. Document IRG/WP/1330. International Research Group on Wood Protection, Stockholm, Sweden.
- Bech-Andersen, J. (1987b) The influence of the dry rot fungus (*Serpula lacrymans*) *in vivo* on insulation materials. *Mater. Organism*. 22:191–202.
- Bech-Andersen, J. (1989) *Serpula lacrymans* the dry rot fungus. Review of previous papers. Document IRG/WP/1393. International Research Group on Wood Protection, Stockholm, Sweden.
- Bech-Andersen, J. Indoor Climate and Mould. Pronoma, Copenhagen, 2004.
- Clausen, C.A., Green, F. (2003) Oxalic acid overproduction by copper-tolerant brown-rot basidiomycetes on southern yellow pine treated with copper-based preservatives. *Int. Biodeterior. Biodegrad.* 51:139–144.
- Clausen, C.A., Kartal, S.N. (2003) Accelerated detection of brown-rot decay: comparison of soil block test, chemical analysis, mechanical properties, and immunodetection. *For. Prod. J.* 53:90–94.
- Connolly, J.H., Arnott, H.J., Jellison, J. (1996) Patterns of calcium oxalate crystal production by three species of wood decay fungi. *Scan. Microsc.* 10:385–400.
- Dutton, M.V., Evans, C.S. (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* 42:881–895.
- Espejo, E., Agosin, E. (1991) Production and degradation of oxalic acid by brown-rot fungi. *Appl. Environ. Microbiol.* 57:1980–1986.
- Gadd, G.M. (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. *Adv. Microb. Physiol.* 41:47–92.
- Green, F., Clausen, C.A. (2001) Oxalic acid production of fifteen brown-rot fungi in copper citrate-treated southern yellow pine. Document IRG/WP/01-10388. International Research Group on Wood Protection, Stockholm, Sweden.
- Green, F., Clausen, C.A. (2003) Copper tolerance of brown-rot fungi: time course of oxalic acid production. *Int. Biodeterior. Biodegrad.* 51:145–149.
- Green, F., Highley, T.L. (1997) Mechanism of brown-rot decay: paradigm or paradox. *Int. Biodeterior. Biodegrad.* 39:113–124.
- Green, F., Larsen, M.J., Winandy, J.E., Highley, T.L. (1991) Role of oxalic acid in incipient brown-rot decay. *Mater. Organism*. 26:191–213.
- Hastrup, A.C.S., Green, F. III, Clausen, C.A., Jensen, B. (2005a) *Serpula lacrymans*, the dry rot fungus and tolerance to copper-based wood preservatives. Document IRG/WP/05-10555. International Research Group on Wood Protection, Stockholm, Sweden.
- Hastrup, A.C.S., Green, F. III, Clausen, C.A., Jensen, B. (2005b) Tolerance of *Serpula lacrymans* to copper-based wood preservatives. *Int. Biodeterior. Biodegrad.* 56:173–177.
- Humar, M., Petric, M., Pohleven, F., Sentjurc, M., Kalan, P. (2002) Changes in EPR spectra of wood impregnated with copper-based preservatives during exposure to several wood-rotting fungi. *Holzforschung* 56:229–238.

- Itakura, S., Hirano, T., Tanaka, H., Enoki, A. (1994) Relationship between degradation of wood, cellulose or lignin-related compounds and production of hydroxyl radical or accumulation of oxalic acid in cultures of brown-rot fungi. Document IRG/WP 94-10062. International Research Group on Wood Protection, Stockholm, Sweden.
- Munir, E., Yoon, J.J., Tokimatsu, T., Hattori, T., Shimada, M. (2001) A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*. *Pros. Natl. Acad. Sci. USA* 98:11126–11130.
- Murray, M.G., Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8:4321–4325.
- Paajanen, L., Ritschkoff, A.C. (1991) Effect of mineral wools on growth and decay capacities of *Serpula lacrymans* and some other brown rot fungi. Document IRG/WP/93-1481. International Research Group on Wood Protection, Stockholm, Sweden.
- Palfreyman, J.W., White, N.A., Buultjens, T.E.J., Glancy, H. (1995) The impact of current research on the treatment of infestations by the dry rot fungus *Serpula lacrymans*. *Int. Biodegrad. Biodegrad.* 35:369–395.
- Palfreyman, J.W., Phillips, E.M., Staines, H.J. (1996) The effect of calcium ion concentration on the growth and decay capacity of *Serpula lacrymans* (Schumacher ex Fr) Gray and *Coniophora puteana* (Schumacher ex Fr) Karst. *Holzfor-schung* 50:3–8.
- Sayer, J.A., Gadd, G.M. (1997) Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger*. *Mycol. Res.* 101:653–661.
- Schilling, J.S., Jellison, J. (2004) High-performance liquid chromatographic analysis of soluble and total oxalate in Ca- and Mg-amended liquid cultures of three wood decay fungi. *Holz-forschung* 58:682–687.
- Schimazono, H., Takubo, K. (1952) The biochemistry of wood destroying fungi: the Bavendamm's reaction and the accumulation of oxalic acid by the wood-destroying fungi. *Bull. Gov. For. Exp. Sta.* 53:117–125.
- Schmidt, O. (2000) Molecular methods for the characterization and identification of the dry rot fungus *Serpula lacrymans*. *Holzfor-schung* 54:221–228.
- Shimada, M., Ma, D.B., Akamatsu, Y., Hattori, T. (1994) A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. *FEMS Microbiol. Rev.* 13:285–296.
- Sutter, H.-P., Jones, E.B.G., Wälchli, O. (1983) The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.) Fr. *Mater. Organism.* 18:241–262.
- Takao, S. (1965) Organic acid production by basidiomycetes. *Appl. Microbiol.* 13:732–737.
- Whitney, K.D., Arnott, H.J. (1987) Calcium oxalate crystal morphology and development in *Agaricus bisporus*. *Mycology* 79:180–187.
- Woodward, B.M., De Groot, R.C. (1999) Tolerance of *Wolfiporia cocos* isolates to copper in agar media. *For. Prod. J.* 49:87–94.

Received December 6, 2005. Accepted February 22, 2006.