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RESEARCH PAPERS

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MICROSCOPIC INVESTIGATIONS CONCERNING *IN SITU* OXALATE FORMATION BY THE BROWN-ROT FUNGUS *PORIA PLACENTA*

Basidiomycete fungi are important organisms for the biodegradation of wood. A main distinguishing feature of many brown-rot fungi is their ability to produce large quantities of oxalic acid. It is well known that the reaction between copper and oxalic acid can result in a detoxification of copper containing wood preservatives, due to the formation of insoluble copper oxalate. However, little information is available regarding the mechanism on a cellular level. The aim of this investigation was to determine in situ copper oxalate formation in order to localise the exact place of precipitation by means of light microscopy and scanning electron microscopy analysis. Additionally, the growth dynamics and minimal inhibitory concentrations on malt extract agar were investigated. Further, energy-dispersive X-ray analysis was used to obtain information about element distribution. Hereby a relative ion concentration not only of copper, but also calcium was measured in the hypha and the surrounding wood tissue. These results are helpful for a better understanding of metabolic strategies in the detoxification of copper in wood impregnated with protective agents.

Keywords: brown-rot basidiomycete, copper oxalate, copper tolerance, *Poria placenta*, *Rhodonia placenta*, wood decay fungi

Introduction

The complex process of the biodegradation of wood caused by basidiomycetes is not fully understood and is still in critical scientific discussion [Green and Highley 1997; Ostrofsky et al. 1997; Schilling et al. 2013]. However, especially

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with regards to copper tolerant wood decay fungi, certain processes and aspects of basidiomycete decay are widely accepted:

a) Brown-rot fungi are growing in an acidic environment [Rayner and Boddy 1988; Humar et al. 2001; Huckfeldt and Schmidt 2015],
b) Production of oxalic acid by wood decay fungi [Espejo and Agosin 1991; Micales 1997; Schilling and Jellison 2005; Fomina et al. 2005; Green and Clausen 2005],
c) Detoxification of copper dissolved in copper-based wood preservatives through copper oxalate formation [Stephan et al. 1996; Sierra-Alvarez 2007; Tang et al. 2013],
d) Mass loss as a quantifiable consequence of wood degradation [DeGroot and Woodward 1999; Clausen and Green 2003; Humar et al. 2004; Hastrup et al. 2012] which leads to considerable decreased strength properties [Pechmann and Schaile 1950; Bari et al. 2016].

Wood decay fungi colonize softwood in radial direction along the wood rays. Then, hyphae penetrate the pits of cross-fields for an additional expansion in axial direction via tracheids. Access to adjacent tracheids is realized by the penetration of bordered pits or by the creation of bore holes [Green et al. 1996]. Brown-rot fungi degrade wood by means of a two-step mechanism, including free radicals and enzymes. Firstly, early degradation is characterised by initial non-enzymatic processes by extracellular produced free oxidative radicals. Secondly, decay continues as a result of enzymatic hydrolytic reactions [Arantes et al. 2012]. The oxidative radicals are generated through Fenton reactions based on peroxides and iron ions in an acidic medium. These radicals are very destructive, have very short half-lives and must be created within the woody cell wall and at a distance from the hyphae. By secretion of organic acids, mainly oxalic acid, the cell wall is acidified. Oxalic acid can chelate iron and these Fe-oxalate complexes can diffuse into the cell wall. Extensive depolymerisation of hemicellulose and cellulose initially occurs in the lignin-poor and cellulose-rich S_2-Layer. In the decay process the tertiary wall (S_3-Layer) remains almost intact in the first instance. The overcoming of the S_3-Layer is associated with free radicals evoked by Fenton chemistry, which depolymerise the cell wall and increase the porosity of the wood structure simultaneously [Eriksson et al. 1990; Arantes et al. 2012]. Subsequently, the surface of the tertiary wall, adjacent to the hyphae, is easier to pass for degrading enzymes and later on also for the hyphae.

The middle lamella maintains pectin with bound calcium [Green et al. 1996]. Calcium is an important component of the middle lamella and assumes the stabilisation function in membranes [Jellison et al. 1997]. The precipitation of calcium oxalate (CaOx) in conjunction with wood decay fungi is described in literature [Goodell et al. 2003]. The transfer of bound calcium into calcium oxalate crystals results in a destabilisation of the middle lamella and a loss of strength of the cell wall for the benefit of the fungi. As mentioned in various

publications, the initial formation of CaOx-crystals by wood decay fungi is under direct control of fungal protoplasts, thus intracellularly. However, the complex mechanism of crystal formation in relation to hyphae remains unclear [Dutton and Evans 1996]. CaOx crystals precipitate as weddellite, whewellite or as a hyphae-coating formation on basidiomycetes [Guggiari et al. 2011].

To protect wooden constructions against fungal decay, wood can be treated with waterborne copper-based preservatives. These formulations include co-biocides such as quats, azoles or Cu-HDO. Effective co-biocides are very important for the protection against copper-tolerant brown-rot fungi. For our studies, we chose copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$) as a model substance and *Poria placenta* (current designation: *Rhodonia placenta* [Jacobs et al. 2016]) as a copper tolerant model fungus. The general colonisation of wood by fungi remains similar, despite preservation treatment as long as the fungicidal concentration of the active agent/s is/are not toxic to the fungi. Furthermore, the way of colonising the wood by wood decay fungi and the way of preservative uptake through wood specimens during the impregnation process are similar [Liese 1975]. *Poria placenta* (*Pp*) is a brown-rot basidiomycete and well investigated as an acknowledged type of the copper-tolerant decay fungi [Clausen et al. 2000; Clausen and Green 2003]. Copper tolerance describes the relative ability of an organism to grow in spite of the presence of a high copper content [Hastrup et al. 2005]. Copper oxalate (CuOx) crystals precipitate mainly as strongly layered insoluble and hence nontoxic moolooite [Tang et al. 2013]. While precipitation of CuOx is well known, little information is available on the biochemical processes of different copper resistance mechanisms in various species [Cervantes and Gutierrez-Corona 1994], and also on how precipitation and initial formation occurs on a cellular level. We investigated the growth dynamics of *Pp* in the presence of copper sulphate and determined the minimal inhibitory concentration (MIC). The major focus was the determination of the *in-situ* precipitation of copper oxalates on a cellular level. Whether copper oxalates or precursor stages, respectively, arise intracellular or extracellular of hyphae still needs fundamental research work.

Materials and methods

Materials

Test fungus, as declared in EN 113:1996, was the brown-rot basidiomycete *Poria placenta* (Fries), isolate FPRL 280/DSM 3088. All tests were carried out with sapwood of *Pinus sylvestris* L. Wood specimens had dimensions of 50 mm × 25 mm × 15 mm, according to EN 113:1996 or of 40 mm × 15 mm × 5 mm (mini blocks) with a similar size to those described by Bravery [1978]. Copper sulphate pentahydrate p.a. ($CuSO_4 \cdot 5\ H_2O$) was solved in different concentrations in demineralised water.

Methods

Specimen preparation for the determination of growth dynamics and MIC was carried out in petri dishes with a diameter of 85 mm containing 4% malt extract and 2% agar (MEA). The copper sulphate solution was added to the autoclaved (121°C, 20 min) MEA-medium and the petri dishes were filled under sterile conditions. The petri dishes containing MEA had copper concentrations of 0, 8, 16, 24, 31 and 47 mM. After solidification, the petri dishes were inoculated with an 8-mm diameter mycelium plug in the middle of the media. The punching out of the mycelium plugs was realised with a cork borer in a concentric way out of the mycelium of the test fungi. Fungal growth was measured and monitored daily for 14 days. The diameter of each mycelium was examined twice from perpendicular measurements. Afterwards the diameter was calculated as the average of these 2 measurements. The execution of the test series was repeated up to 9 times. The MIC range covers 2 values and was defined on day 7 of each experimental run. The first value is the highest Cu-concentration tested, at which hyphae growth is not suppressed. The second value is the lowest tested Cu-concentration which completely eliminates hyphae growth.

Specimens for light microscopy, field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray analysis (EDXA) were prepared from wood specimens with two different volumes (3.00 cm^3, 18.75 cm^3) and 2 different retentions of Cu in each case (Cu 0.7 kg/m, Cu 1.4 kg/m^3) as well as untreated controls. The impregnation of wood specimens was carried out according to EN 113:1996. After treatment, the Cu uptake corresponded to a salt retention of 2.63 kg/m^3 and 5.38 kg/m^3 $CuSO_4 \cdot 5\ H_2O$. Afterwards treated and untreated EN 113 specimens were positioned on MEA (4% malt extract, 2% agar) lying on a ring of glass in Kolle flasks. In every Kolle flask 2 samples with the same treatment were located and between specimens MEA was inoculated with an 8-mm mycelium plug. The same preparation was done with 3.00 cm^3 specimens in petri dishes, but without glass rings. Kolle flasks and petri dishes were incubated in a climate chamber with 70 ±5% relative humidity and 22 ±2°C for 6 weeks. Exposition time was based on the screening method [Bravery 1978]. Investigations to evaluate mass loss were executed from specimens with the scale AX 204 (Mettler-Toledo GmbH, Gießen, Germany).

Light microscopy analysis was performed with a transmitted light microscope Axio Lab. A1 equipped with a camera AxioCam 105 colour (both from Carl Zeiss AG, Oberkochen, Germany). Microtome sections (15 µm thick) were cut from mini blocks (V = 3 cm^3) with the microtome SM 2400 (Leica Microsystems GmbH, Wetzlar, Germany). Radial and tangential sections were cut from a) front surface sections and b) from the middle zones of specimens referring to the axial orientation of wood. Hereby, all microtome sections from the front surface and middle zones were less than 2 mm off the wood surface.

Finally, image processing and analysis was performed using the programme Zen lite 2012 (Carl Zeiss AG, Oberkochen, Germany).

Measurements of specimen surfaces were executed with a FESEM Quanta FEG Type 250 (FEI, Eindhoven, Netherlands). Selected FESEM samples were dried overnight at 60°C in an oven. After sputter coating with gold for approx. 165 seconds with the coating system SC 510 (Bio-Rad Microscience Division, Hercules, California, USA), samples were prepared for FESEM. For element analysis samples were coated with carbon before being examined by a scanning electron microscope SEM S-520 (Hitachi Ltd. Corporation, Tokyo, Japan) equipped with an energy dispersive X-ray device EDX eumex Si(Li)-detector (EUMEX GV, Mainz, Germany). Element measurements were performed with an excitation energy of 10 keV.

Results and discussion

Growth dynamics

The growth dynamics of *Poria placenta* (*Pp*) mycelium on media supplemented with different amounts of Cu^{2+} ions are presented in figure 1. All graphs represent the mean function of the test series and start at 8 mm diameter due to the inoculation plug size. Measurements are restricted to 85 mm diameter of petri dishes. No correlation was found between the age of mycelium of the inoculation plug and the growth vitality of *Pp*. The test procedure was replicated from 2 to 9 times. The applied gradation of preservative concentrations is optimized for *Pp* growth dynamics on copper sulphate, based on results generated in the previous test series. Curve progressions of 0, 8, 16 mM Cu graphs display sigmoid properties (fig. 1).

Initial phases of respective curves are monotonously rising with progressive growth. Abating phases are likewise monotonously rising, hence with a degressive growth, as seen on the 16 mM Cu graph. Both phases meet at the turning point of the idealised sigmoid function. This point represents the middle of the sector with the most vital growth of mycelium. The radial growth of the control was about 5 mm/day at a temperature of 21°C. As expected, the higher the Cu-concentration in MEA, the more inhibition is related to mycelial growth of *Pp*. Based on the expansion of mycelium growth on day 7 the MIC range is derived as 24-31 mM Cu corresponding to 1500-2000 ppm Cu (fig. 1). The second MIC value correlates well with the toxicity value of 32 mM, as published by Sierra-Alvarez [2007].

Weight loss

EN 113 specimens and mini blocks were exposed to *Pp* for 6 weeks. Figure 2 illustrates that an increasing sample volume results in a lower weight loss on

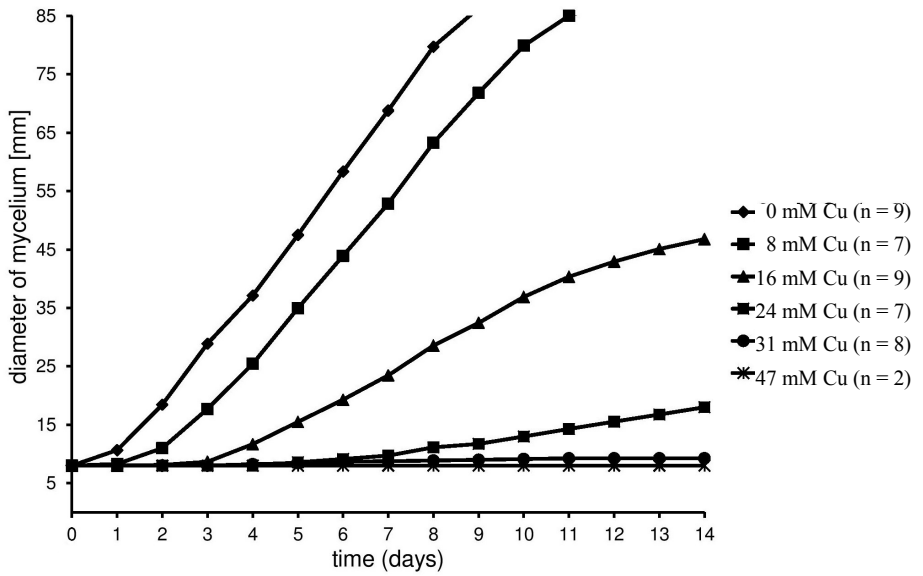

Fig. 1. Mean growth dynamics of *Poria placenta* FPRL 280/DSM 3088; MEA treated with different concentrations of copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$); n = number of replicates

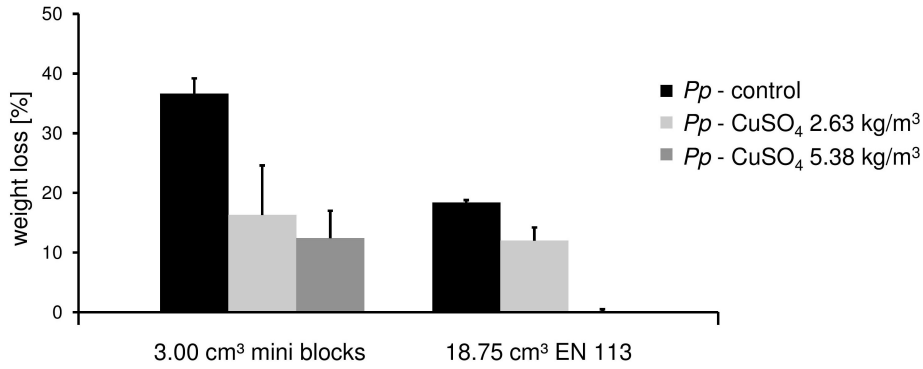

Fig. 2. Weight loss after 6 weeks from different volume samples of *Pinus sylvestris* L. treated with 2 different concentrations of copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$) and untreated controls, *Pp - Poria placenta*; n=4

a percentage basis, due to a different volume-surface-ratio in relation to the colonisation of sapwood by basidiomycetes. As expected, controls have the highest amount of weight loss in both setups. The mean mass loss of 18.4% of EN 113 control samples correlated to results from Wälchli [1977]. He reported a weight loss of 21.5% after 8 weeks exposure of *Pinus sylvestris* L. EN 113

specimens to *Pp*. Treated EN 113 samples with a retention of 5.38 kg/m^3 $CuSO_4 \cdot 5\ H_2O$ showed no signs of decay by *Pp*, whereas mini blocks were decayed to a mass loss of 12% (fig. 2). This result also displays the influence of different volume-surface-ratios and thickness of test blocks on the results.

Precipitation of copper oxalate

Microscopical analysis was only carried out on mini blocks. These samples showed higher mass losses for each treatment and therefore, a higher amount of CuOx precipitation was expected in these specimens. In specimens treated with copper sulphate, precipitation of CuOx (moolooite) crystals as well was CaOx (weddellite) was found within the wood matrix (light microscopy) and on the surface of the wood specimens (FESEM/EDXA). Furthermore, destruction of the wood matrix (cracks, bore holes) and penetration of bordered pits and fenestriform pits was observed.

Light microscopy

In the front surface sections of treated samples CuOx and CaOx crystals were found in wood rays and in tracheids, whereas only CaOx (as weddellite) was observed in the front surface sections of the controls (tab. 1). In the middle zones of samples with low copper sulphate retentions CuOx and CaOx crystals were found in rays and tracheids located near the surface. Contrasting, in the middle zones of sections with high copper sulphate uptake CuOx could only be found in wood rays, but not in tracheids. That leads to the conclusion that *Pp* was not able to expand axially via tracheids within 6 weeks of exposition time. However, initial radial growth had already started, since CuOx was found in rays of the middle zones (tab. 1).

Table 1. Summary of screening of crystals under the light microscope (mini blocks inoculated for 6 weeks with *Poria placenta* (*Pp*))

	Front surface				Middle zone			
	wood rays		tracheids		wood rays		tracheids	
	CuOx	CaOx	CuOx	CaOx	CuOx	CaOx	CuOx	CaOx
Pp control	–	+	–	+	–	×	–	×
Pp 2.63 kg/m^3 $CuSO_4$	+	+	+	+	+	+	+	+
Pp 5.38 kg/m^3 $CuSO_4$	+	+	+	+	+	–	–	–

– no finding + finding × no image available

The strong inhibition of axial expansion is most likely due to a higher concentration of the fungicide acting copper sulphate pentahydrate in the middle zone. Depending on specimen dimensions and formulation investigations on the

distribution of copper in softwood treated with copper based preservatives prove a considerably elevated concentration of copper in wood rays and adjacent tracheids as well as in cell corners, especially in the outermost zones [Evans et al. 2013]. Moreover, according to Liese [1975] copper tolerant fungi maintain their colonisation strategies despite the presence of copper based preservative when in a non-toxic concentration. Hence, in order to grow, *Pp* would have to overcome sample areas with the highest copper content. That leads to the conclusion that "natural ways" of fungal colonisation contain the highest amount of copper and copper tolerant fungi like *Pp* have to overcome this restriction. This is why in our investigations, growth expansion of *Pp* was massively inhibited in the first steps of radial expansion in wood rays in the middle zone. On the samples front surface, *Pp* was able to penetrate tracheids in a tracheid-to--tracheid manner. Consequently, in this direction degradation of wood by fungi was clearly less restricted in respective xylem tissue.

In microtome sections from specimens with 2.63 kg/m^3 salt retention, wood rays and cross-fields were massively degraded. Thus, oxalate crystals precipitated in wood rays during degradation, now appear in adjacent tracheids (fig. 3a). The occurrence of CaOx-formation in accordance with fungal hyphae growth, first appears in ray cells before expanding into tracheid lumina (fig. 3a).

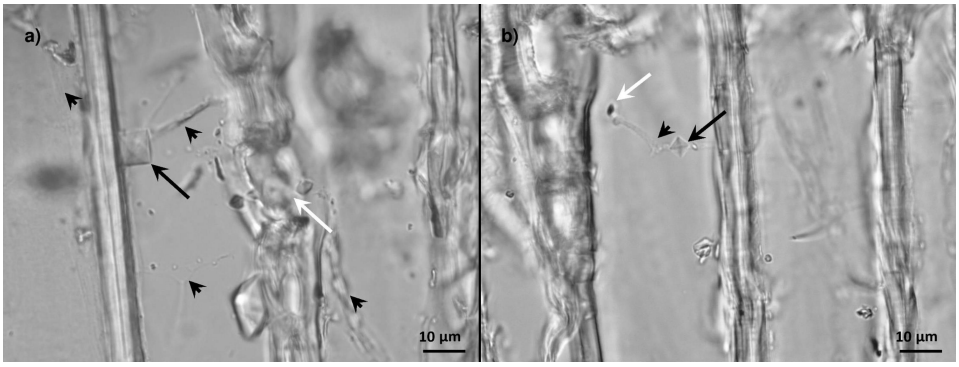

Fig. 3. Light microscopy images (tangential section) from *Pinus sylvestris* L. containing 2.63 kg/m^3 copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), inoculated with *Poria placenta* and incubated for 6 weeks; CaOx formation (black arrow); hyphae (black arrowheads); a) highly degraded wood ray (white arrow); b) bore hole in tracheid cell wall (white arrow)

Also, direct contact of CaOx to hyphae was observed more frequently than direct contact of CuOx to hyphae (fig. 3b). However, exact localisation of oxalates by light microscopy methods remains difficult. No definite evidence could be found to determine whether oxalate crystals were enclosed by hyphae membrane or located outside of the hyphae. Occurrences of vesicular swellings on hyphae have been described by Huckfeldt and Schmidt [2015] for the habitus

of *Pp*. These swellings were frequently observed in our treated and untreated samples. Mostly, those vesicular swellings appeared to be burst (fig. 4).

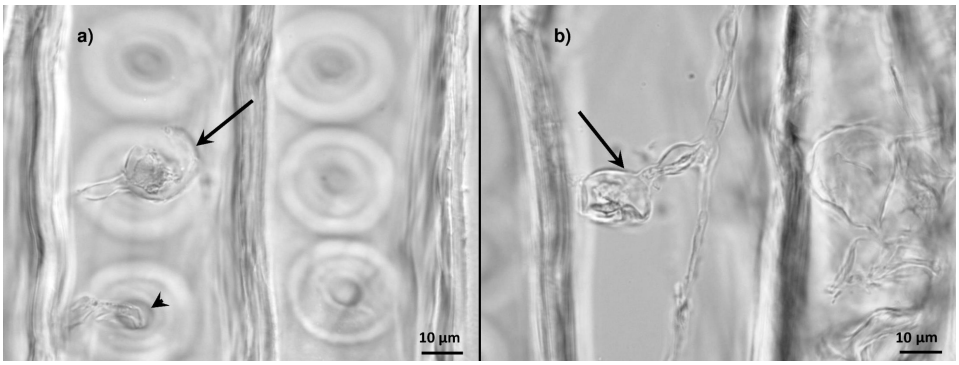

Fig. 4. Light microscopy images (radial section) from *Pinus sylvestris* L. containing 5.38 kg/m^3 copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), inoculated with *Poria placenta* and incubated for 6 weeks; a) and b) burst vesicular swellings (black arrows); a) penetration of bordered pit by hypha (black arrowhead)

Additional investigations were performed on MEA containing copper sulphate. Within a few days after inoculation, a distinct zone of solubilisation surrounding the mycelium was observed. Subsequently, concentric ring-shaped areas were formed. These areas differed in colour to the homogenously coloured MEA. Light microscopic investigations confirmed the precipitation of CuOx crystals in these concentric areas. CuOx crystals were not observed in the zone in front of the hyphal growth frontier. The formation of the crystals occurred behind the growth border with temporal offset of 1 to 2 days.

Field emission scanning electron microscopy – FESEM

Analysis with light microscopy on sample sections gave revealing insights into the strategy of fungal colonisation and precipitation of oxalates in relation to wood degradation by copper tolerant fungi. However, this method is restricted in terms of localising intracellular or extracellular precipitation of oxalates. Artificial translocation and washout of oxalates and hyphae is possible due to sample preparation. Using FESEM technique the localisation of the copper oxalate crystals can be observed in more detail. We observed that CuOx crystals were located at the hyphae, sometimes forming microcrystalline structures that enclosed the hyphae (fig. 5). It appeared that the crystals were embedded in the mucilaginous sheath surrounding the hyphae. Similar findings were also reported by Dutton and Evans [1996]. We also found the contents of burst vesicular swellings (fig. 6). The contents found inside those swellings of hyphae appeared to be strongly layered, which indicates possible CuOx formation

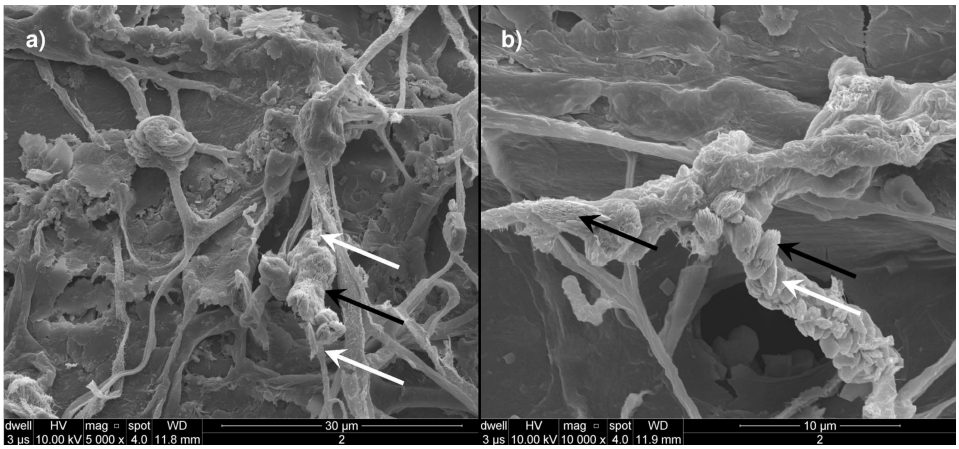

Fig. 5. FESEM images on surface of *Pinus sylvestris* L. containing 5.38 kg/m^3 copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), inoculated with *Poria placenta* and incubated for 6 weeks; a) and b) microcrystalline CuOx-structures (black arrows) coating hyphae (white arrows)

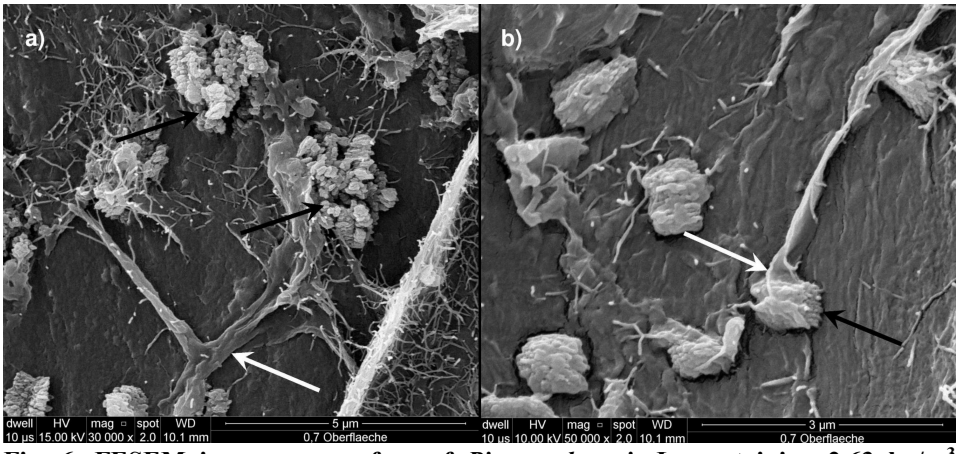

Fig. 6. FESEM images on surface of *Pinus sylvestris* L. containing 2.63 kg/m^3 copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), inoculated with *Poria placenta* (*Pp*) and incubated for 6 weeks, CuOx formation and burst vesicular swellings of hyphae of *Pp*; a) hyphae network (white arrow) with strongly layered oxalates on ends of burst hyphae (black arrows); b) end of hypha (white arrow) with partly uncovered CuOx (black arrow)

(fig. 6). Similar findings of formations in hyphae of metal tolerant fungi were described by Fomina et al. [2005]. These structures also appeared to be layered crystals. It is possible that excessive secretion of oxalic acid will lead to crystallisation within the fungal cell wall resulting in the hyphae bursting as well. Morphology and growth of crystals are dependent on factors like time, fungal species, ambient medium and pH level. Investigations on CuOx-

nanocrystals demonstrated various anisotropic characteristic forms depending on time and ambient media [Romann et al. 2009].

Energy-dispersive X-ray analysis – EDXA

Detecting CuOx precipitations by EDXA was performed using element mapping of copper. Thereby, several oxalates were detected in direct contact to hyphae (fig. 7). The present results do not define precisely if precipitation takes place intracellular or extracellular of fungal hyphae of *Pp*. However, the future emphasis of research should focus on the elemental analysis of Cu inside and outside hyphae in order to gain deeper insights into fungal physiology during CuOx precipitation. In this process, special emphasis should be placed on the contents of vesicular swellings of the hyphae of *Pp*. These investigations would help to clarify the crystalline formations we observed at burst vesicular swellings, as shown in figure 6b.

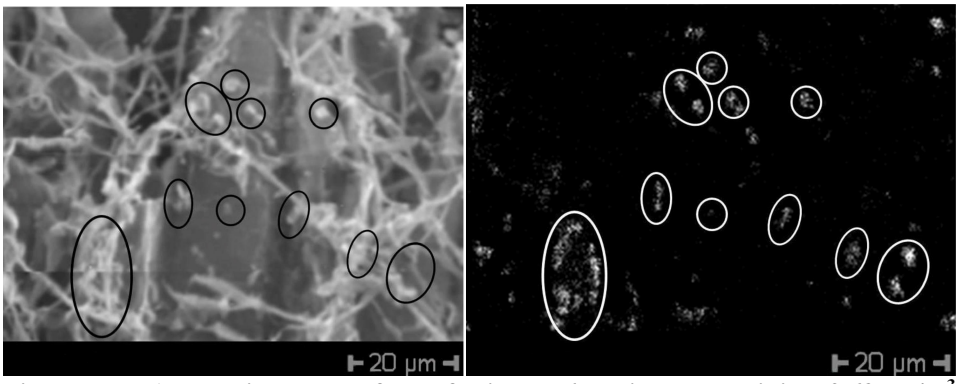

Fig. 7. EDXA mapping on surface of *Pinus sylvestris* L. containing 2.63 kg/m^3 copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), inoculated with *Poria placenta* and incubated for 6 weeks; left: SEM-image with selected areas with verified Cu (black ellipses) and right: elemental analysis of SEM-image according to Cu in selected areas (white ellipses)

Intracellular chelation of copper by metallothioneins in fungal cells has also been described by Cervantes and Gutierrez-Corona [1994]. Taking all this into consideration, questions on the physiology of copper tolerant fungi arise: which other mechanisms besides copper oxalate formation confer copper tolerance to brown-rot basidiomycetes like *Poria placenta?*

Furthermore, possible effects of the sulphate ions present in our experiments have to be taken into consideration in relation to the respective fungal strategy. Copper tolerant fungi are known to be able to precipitate copper from dissolved copper based preservatives into insoluble non-toxic CuOx. Therefore, the amount of toxic acting copper cations (Cu^{2+}) does decrease. However, sulphate anions are still active in this complex process. According to this, fungi may well

be copper tolerant, but other compounds (e.g. SO_4^{2-}) of the preservative may cause lethal toxicity to basidiomycetes [Leithoff and Melcher 1999].

The production of oxalic acid plays an important role in the wood degrading process. Oxalic acid is the basis for many chemical reactions like the Fenton reaction [Green et al. 1991] and is involved in CuOx-precipitation [Murphy and Levy 1983] during wood cell wall degradation. However, the correlation of oxalic acid production and copper tolerance is not linear [Clausen et al. 2000], but given as a clear tendency [Sierra-Alvarez 2007]. The precise amount of oxalic acid, that is actually needed for degradation as well as how much oxalic acid a fungus is able to produce is yet not fully understood.

Conclusions

Precipitation of calcium oxalates in *Pinus sylvestris* L. caused by *Poria placenta* occurs in the course of wood degradation. A higher uptake of copper sulphate in *Pinus sylvestris* L. leads to less mass loss caused by *Poria placenta*. Parallel to the extracellular formation of copper oxalates, our investigations indicate the precipitation of copper oxalate within hyphae in vesicular swellings.

Using various microscopically techniques (light microscopy, FESEM, EDXA) oxalate formations were identified within the wood. For a better understanding of copper tolerance in *Poria placenta*, focused on the precise localisation of copper oxalate crystals, further research is needed.

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List of standards

EN 113:1996 Wood preservatives – Method of test for determining the protective effectiveness against wood destroying basidiomycetes – Determination of the toxic values

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