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Sterols and lignin in *Eucalyptus globulus* Labill. wood: Spatial distribution and fungal removal as revealed by microscopy and chemical analyses

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Abstract

Wood decay experiments were carried out aiming at the selective removal of lipophilic compounds with selected basidiomycetes isolated from *Eucalyptus globulus* plantations in Uruguay: *Dendrophora albobadia*, *Lentinus tigrinus*, *Peniophora cinerea*, *Peniophora lycii*, and *Phanerochaete crassa*. Localization and composition of lipophilic compounds and lignin of *E. globulus* were determined by gas chromatography-mass spectrometry, fluorescence microscopy using filipin staining, confocal laser scanning microscopy (CLSM), and low temperature scanning electron microscopy. Free and esterified sterols, mainly sitosterol, were the predominant lipophilic compounds in the control wood. Sterols were present in ray parenchyma cells, together with polyphenols, and in vessels. This confirms earlier observations indicating that these cell types are the principal source of lipophilic extractives involved in pitch problems during pulping and bleaching. Sterols are also present in the vestures of fiber and vessel pits. Different fungal degradation patterns of *E. globulus* wood were determined. *P. lycii* showed the highest specificity for lignin degradation during short incubation time together with considerable sterol removal capacity. Ray parenchyma cells and their lumen deposits were strongly degraded by *P. lycii*. Eucalypt lignin located in vessel walls and fiber cell corners was more resistant to fungal attack, as revealed by CLSM. The initial decay stage of *L. tigrinus* was restricted to vessels and tyloses where the sterol compounds were removed.

Keywords: basidiomycetes; biopulping; confocal laser scanning microscopy (CLMS); fluorescence microscopy (FM); low temperature scanning electron microscopy (LTSEM); microscopy; pitch; sterols.

Introduction

Extractives are known to be important for physical and chemical properties of wood and provide in special cases resistance against fungal decay (Hillis 1987). The amount and localization of wood extractives and the morphological features of the cells that contain these compounds affect the industrial deresination of this material (Back and Allen 2000). The chemical and morphological behavior of the extractives during pulping also affects industrial bleaching (Fernando et al. 2005; Freire et al. 2006). Free and esterified sterols present in eucalypt wood can survive totally chlorine free (TCF) bleaching process of kraft pulps and have been identified as being responsible for pitch deposition (Gutiérrez et al. 1998). In this regard, a promising biotechnological approach has been reported based on the ability of certain basidiomycetes, such as *Ceriporiopsis subvermispora* and *Phlebia radiata*, to degrade free and esterified sterols in eucalypt wood. The process is known as biodepitching (Gutiérrez et al. 1999; Martínez et al. 1999).

In spite of the importance of non-saponifiable sterols in pitch deposits, little information is available about their spatial distribution in hardwoods and pulps. Filipin, a fluorescent polyene antibiotic, has been used as a tool to determine the spatial localization and redistribution of sterols during manufacturing of TCF pulps from eucalypt and birch (Speranza et al. 2002; Fernando et al. 2005). It is also suited to evaluate the efficiency of laccase mediators applied in the course of sterol removal from TCF bleached eucalypt pulp (Gutiérrez et al. 2006; Speranza et al. 2007).

Lignin is the more recalcitrant plant polymer and its mineralization by white rot basidiomycetes plays a major role in carbon recycling (Martínez et al. 2005). The unique ability of white rot for lignin degradation is used in biopulping. It is a solid substrate fermentation (SSF) process where lignocellulosic materials are treated with selected ligninolytic white rot fungi, such as *C. subvermispora* and *Phanerochaete chrysosporium*, to improve pulping (Akhtar et al. 1998). The chemical and physical modifications of wood generated by biopulping provide better pulp properties and reduce cooking time and refining energy (Akhtar et al. 1998; Hunt et al. 2004; Mendonca et al. 2004). Besides that, the biopulping effect can also be achieved by *Ophiostoma piliferum*, a fungus that degrades extractives but not lignin and improves the penetration of pulping liquors (Farrell et al. 1993).

The ability and strategies of fungi to decay lignin and cellulose is well characterized (Daniel 1994; Blanchette 1995). However, studies are scarce concerning the decay abilities of fungal communities living on eucalyptus (Bettucci et al. 1998; Ferraz et al. 1998; Speranza 2003).

In addition, information about the degradation of non-structural components in hardwood, such as eucalyptus, by white rot fungi is limited (Gutiérrez et al. 1999; Martínez et al. 1999).

In the present work, the topochemistry of lignin and lipophilic extractives was studied and involved those extractives responsible for pitch problems. *Eucalyptus globulus* wood was treated with selected fungal species and strains, isolated from *Eucalyptus* (Speranza 2003), aiming at the removal of the lignin and extractives. Gas chromatography-mass spectrometry (GC-MS), fluorescence microscopy (FM) using filipin staining, confocal laser scanning microscopy (CLSM), and low temperature scanning electron microscopy (LTSEM) were the methods of choice.

Materials and methods

Fungal species

Basidiomycetes investigated: *Dendrophora albobadia* (Schwe.) Chamuris IJFM A787, *Lentinus tigrinus* (Bull.) Singer IJFM A503, A768 and A769, *Peniophora cinerea* (Fr.) Cooke IJFM A791, *Peniophora lycii* (Pers.) Höhn. & Litsch. IJFM A745 and *Phanerochaete crassa* (Lév.) Burds. IJFM A744. Stock cultures were maintained on malt agar slants in the dark at 4°C at the IJFM culture collection (CIB-CSIC, Madrid).

Wood samples and in vitro decay experiments

Industrial chips (≈4×20×20 mm) from 14-year-old *E. globulus*, supplied by ENCE (Pontevedra, Spain), under water saturation conditions were investigated in SSF experiments. Decay experiments were carried out by placing six previously aseptically sterilized chips on the surface of 7–10-day-old fungal colonies, pregrown on 2% malt extract agar in Petri dishes. Decay dishes, three for each fungus, were incubated in the dark at 28°C and 80% constant humidity. After 14 and 28 days of incubation, the mycelium was manually removed from wood chips and the samples stored at -20°C until chemical and microscopical analyses. Non-inoculated wood chips served as control.

Chemical analyses

Wood samples were dried at 100°C to constant weight to determine the water content, and the weight loss was determined in base of the initial and final dry weights. The weight loss resulting from sterilization (1.0±0.2%) was taken into account when the weight loss due to biodegradation was determined (Guerra et al. 2004). All chemical analyses were performed on 40–60 mesh wood meal samples (Tappi 1996–1997). The milled wood was dried at 60°C in an aerated oven for 12 h, and extracted with acetone in a Soxhlet for 8 h (Tappi 1996–1997). The extract was dried for weight estimation and redissolved in 500 μl of chloroform to be analyzed by GC-MS (Gutiérrez et al. 1998). Compounds were identified and quantified by their area using the Wiley and Nist libraries and calibration curves of standards. The Klason lignin was determined according to Effland (1977). A specificity factor, calculated as the ratio between lignin loss and wood weight loss, was used for detecting fungi with selective lignin degradation.

Microscopy analyses

Anatomical characterization was carried out by LTSEM. Hydrated wood cryo-sections were mounted with cryo-glue O.C.T. (Gurr, BDH, Poole, UK) on specimen holders and plunged into subcooled LN_2 with a cryo-transfer system (Oxford CT1500 HF). Samples were etched (-90°C for 2 min) in the cryo-preparation chamber, gold sputter-coated under vacuum (4×10^{-1} torr), and observed using a Zeiss DSM960 microscope (CCMA, CSIC, Madrid), -135/-150°C, operated at 15 kV at varying working distances.

Lignin and extractives autofluorescence from wood cryo-sections (20–25 μm thick) was detected by CLSM (Bio-Rad MRC 1024) using an Argon/Krypton laser (wavelength, 488 and 514 nm). Images were captured at green and red wavelengths with a band pass filter 540 DF30 and the 580 DF32, respectively. A series of confocal images were collected at a resolution of 1024×1024 pixels, and for each single image 15–20 scans were taken. For final images, the green and red channels were combined (Laser Sharp processing from Bio-Rad software version 3.2).

Filipin, a fluorescent antibiotic reacting specifically with 3β-hydroxysterols, including sitosterol, was the stain used to determine the spatial distribution of free sterols in eucalypt by FM (Speranza et al. 2002). The wood cryo-sections (7–10 μm thick) were stained and observed by phase contrast and epifluorescence with an Axioplan Zeiss microscope. Cryo-sections from non-incubated wood were extracted in a Soxhlet apparatus with acetone during 8 h and washed with water, and served as extractive free control.

Results and discussion

Fungal removal of lignin and lipophilic extractives from *E. globulus* wood

The results concerning losses of weight, lignin, and sterols (free and esterified) caused by the different fungal strains, as well as the fungal specificity for lignin degradation are presented in Table 1.

After 14 days, the weight loss values ranged from 2±2% (*P. lycii*) to 6±2% (*P. cinerea*). After 28 days of incubation *L. tigrinus* A768 produced the highest weight loss (11±2%), whereas *P. lycii* produced the lowest weight loss (5±3%).

Some fungal species show specificity for lignin degradation (Blanchette 1995). Also, the fungal strains investigated in the present work possess high specificity for lignin degradation during SSF experiments of 60 days (del Río et al. 2002; Speranza 2003). Seven selected fungi degraded lignin with high specificity after 28 days of incubation. Only *P. lycii* was effective with this regard already in the initial stages of decay. This fungus also produced the highest lignin degradation (20±3% and 61±21%) and revealed the highest specificity (10.0–12.2) in the 14 and 28 days of incubation periods. Different lignin degradation rates for *L. tigrinus* strains were found, and the isolate A768 showed the greatest specificity for lignin removal (1.3) similar to that demonstrated by *P. crassa* after 14 days of incubation.

Regardless of the selective removal of lignin by white rot fungi, the substrate is not uniformly degraded. The preferential lignin degradation occurs in patches (Blanchette 1995; Ferraz et al. 2000). The reason is the inhomogeneity of wood consisting of various cell types with different distributions. The chemical composition of the cell wall is not homogeneous either (Watanabe et al. 2004). These facts explain the 21% standard deviation

Table 1 Degradation of *E. globulus* wood components after fungal treatments and fungal specificity to remove lignin.

Species/strains	Incubation time (days)	Percentage of degradation[a]				S[d]
		Weight	Lignin	Sterols[b]	Sterols esters[c]	
Dendrophora albobadia A787	14	6 (0)	5 (5)	63	67	0.8
	28	7 (4)	23 (1)	–	–	3.3
Lentinus tigrinus A503	14	5 (2)	1 (3)	29	38	0.2
	28	8 (2)	16 (2)	–	–	2.0
Lentinus tigrinus A768	14	4 (1)	5 (1)	38	48	1.3
	28	11 (2)	25 (3)	–	–	2.3
Lentinus tigrinus A769	14	5 (3)	0 (3)	71	84	0.0
	28	7 (2)	17 (3)	–	–	2.4
Peniophora cinerea A791	14	6 (2)	1 (3)	69	57	0.2
	28	9 (1)	37 (3)	–	–	4.1
Peniophora lycii A745	14	2 (2)	20 (3)	23	88	10.0
	28	5 (3)	61 (21)	–	–	12.2
Phanerochaete crassa A744	14	4 (0)	5 (13)	20	10	1.3
	28	8 (0)	33 (2)	–	–	4.1

[a]Degradation losses in relation to their original content in wood control, mean and standard deviation. [b,c]Sterols and sterol esters removal were determined in the 14 day inoculated wood samples. [d]Specificity calculated as the ratio between lignin loss/weight loss. *E. globulus* wood control content: lignin 20.3%, 0.8% of acetone extractive, sterols and 29 mg/100 mg of wood and esterified sterols 21 mg/100 mg of wood.

of lignin loss found in the replicates of wood decay in the case of *P. lycii* A745 after 28 days.

A representative chromatogram of the lipophilic extractives from the control is presented in Figure 1. Free fatty acids (palmitic, linoleic, oleic and stearic), squalene, ketones and steroid hydrocarbons, free and esterified sterols, and small quantities of triglycerides were identified. This is in agreement with previous reports (Swan and Åkerblom 1967; Gutiérrez et al. 1998). Free and esterified sterols, mainly sitosterol, which represent 75% of the total sterols, were the predominant lipophilic extractives, amounting to 34% and 24% of the chromatographied compounds, respectively. These sterols are responsible for pitch deposition during eucalyptus TCF pulp production (Gutiérrez et al. 1998). Their removal by the different fungal strains was analyzed after 14 incubation days. GC-MS analyses of the lipophilic extractives showed that all seven fungal strains tested were able to degrade most of these compounds, though at different rates (Table 1).

L. tigrinus A769 achieved the greatest removal of both free and esterified sterols (71–84%). *D. albobadia* and *P. cinerea* removed more than 50% of the steroids. The ligninolytic activities of the three fungi were nearly negligible in this incubation time. On the other hand, *P. lycii* – the fungi with the highest lignin specificity – degraded esterified sterols at similar rates as *L. tigrinus* A769. Some selected chromatographic profiles of strains with a high lignin specificity factor are shown in Figure 1 corresponding to *L. tigrinus* A768, *P. lycii*, and *P. crassa*.

Martinez et al. (1999) reported that the basidiomycetes *C. subvermispora* and *P. radiata* can be used for the biological control of lipophilic compounds in the course of paper pulp manufacture from hardwoods. The basidiomycetes selected in this work could degrade problematic lipophilic compounds in similar percentages as *C. subvermispora* and *P. radiata*, and were also able to degrade lignin efficiently. In this context, *P. lycii* is the best fungus.

An external source of nutrients, such as malt extract, enhances further fungal decay activity (Boyle 1998). However, fungal growth promoters for the improvement of colonization of wood chips in large-scale biopulping trials increase the risk of culture contamination by other microorganisms (Akhtar et al. 1998). In this context, the ability of the selected white rot fungi to compete effectively with indigenous fungi in wood is essential (Akhtar et al. 1998). The physiological and ecological characteristics of *P. lycii* may facilitate a successful colonization of eucalypt chip piles in field experiments since it is able to grow at different water potentials and it is a dominant species in wood rotting fungal communities (Rayner and Boddy 1988; Griffith and Boddy 1991). Their natural occurrence in eucalyptus trees and wood may be an advantage for colonization on this substrate (Speranza 2003).

Micromorphology aspects of eucalypt wood degradation

Wood decay by *L. tigrinus* A768, *P. lycii*, and *P. crassa* were analyzed after 14 day treatments with specific microscopic techniques: 1) LTSEM to observe structural changes in wood, 2) CLSM to localize lignin degradation, and 3) FM using filipin staining to detect sterol removal.

Uniseriate fibers, parenchyma rays, and solitary vessels of medium size were the predominant cells observed in *E. globulus* wood by LTSEM (Figure 2a, d and e). Ray parenchyma cells contained abundant spherical deposits (Figure 2a). Vestured pits, consisting of bordered pits with the pit aperture filled with projections from the secondary cell wall (vestures), were observed in fibers and vessels (Figure 2b). Similar vestures have also been reported in *Eucalyptus regnans* (Scurfield and Silva 1970) and *Eucalyptus camaldunensis* (Watanabe et al. 2006). Morphological and chemical features of vestured pits confer important technical properties to wood and affect its industrial processing (Back and Allen 2000). These structures allow the transport of water through intervessel pits and could also increase hydraulic resistance and prevent pit membrane rupture in vessels, fibers, and ray parenchyma cells (Sano 2005; Watanabe et al. 2006). When wood was acetone extracted structural modifications of

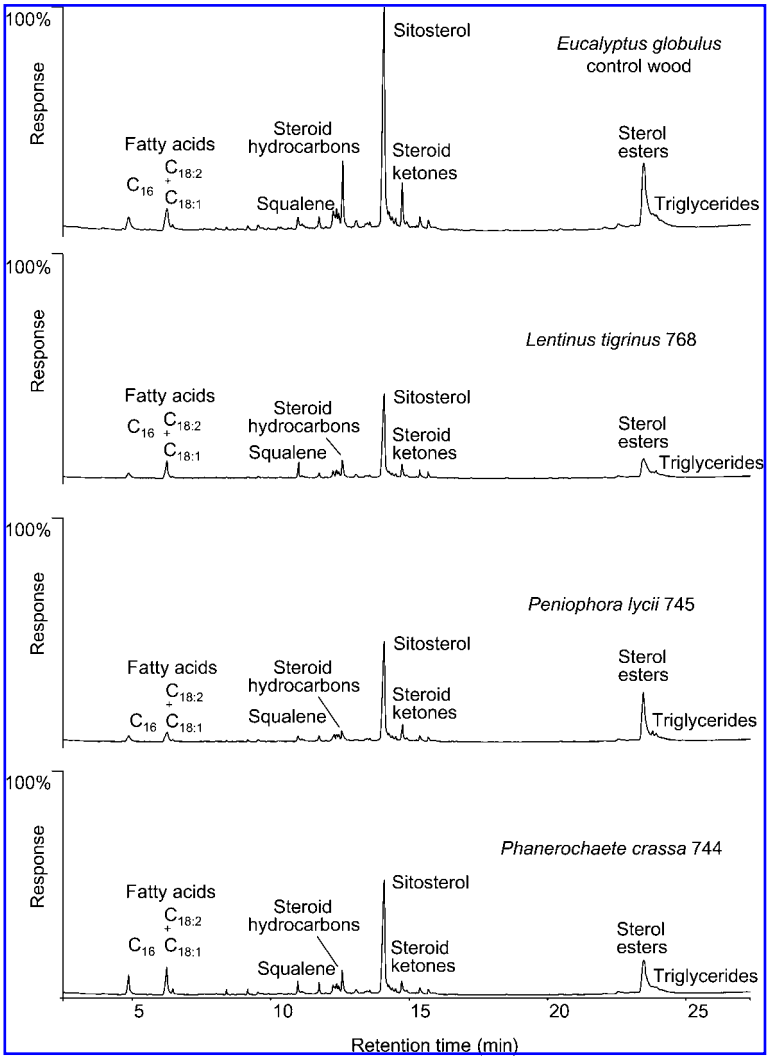

Figure 1 Gas chromatography-mass spectrometry analysis of lipophilic extractives of *E. globulus* wood after 14 days of incubation with *L. tigrinus*, *P. lycii*, and *P. crassa* and the respective non-inoculated control.

vestures were observed by LTSEM, probably due to the removal of polar and apolar extractives (Figure 2c).

After 14 days of incubation, *E. globulus* wood was colonized by *L. tigrinus* A768, *P. lycii*, and *P. crassa* (Figure 2d–f), although different degradation patterns were observed by LTSEM. *L. tigrinus* hyphae colonized vessels extensively (Figure 2d). Tyloses, which act as barriers to microorganisms and helps to confer durability because of its chemical composition (Hillis 1987), were penetrated by *L. tigrinus* hyphae (Figure 2d). However, ray parenchyma cells and fibers remained unaltered during the incipient stages of decay. *P. crassa* produced a more extensive colonization and hyphae were observed in vessels and parenchymatic rays (Figure 2e). Some spherical deposits from parenchymatic rays disappeared in wood degraded by *P. crassa*, although these cells remained relatively unaltered (Figure 2e). *P. lycii* produced abundant fungal mycelium in parenchymatic rays, which were severely degraded and led to the disappearance of deposits, and left the fibers slightly modified (Figure 2f).

Lignin from plant cell walls emits autofluorescence with blue or green light excitation that can be directly detected by CLSM, thus providing information about its distribution and relative concentration. Although holocellulose emitted a weak autofluorescence under the CLSM conditions used, the green fluorescence observed in wood samples is mainly due to lignin (Donaldson et al. 2001). In addition, a strong yellow autofluorescence was detected in spherical deposits and walls from parenchyma cells and vessels in control wood (Figure 3a–c). This fact may be explained by the different chemical composition of wood polyphenols in vessels and ray parenchyma cells that could interfere with the absorption spectrum of lignin (Hillis and Sumimoto 1989; Watanabe et al. 2004). On the other hand, the oxidative degradation of side-chain units in lignin due to fungal enzymes generates quinones and produces the lignin autofluorescence loss which can be detected by CLSM (Barsberg et al. 2003).

The wood treated with *L. tigrinus* A768 showed strong autofluorescence in fibers, ray parenchyma cells walls, and deposits (Figure 3d), similar to that found in the control wood when analyzed with LTSEM. Accordingly, its limited ability to degrade lignin during short-time wood chip treatment is confirmed (Table 1). On the other hand, *P. crassa* caused the removal of spherical deposits in lumens of parenchymatic rays and this led to loss of autofluorescence (Figure 3e). However, this fungus did not reveal lignin degradation during the incipient stages

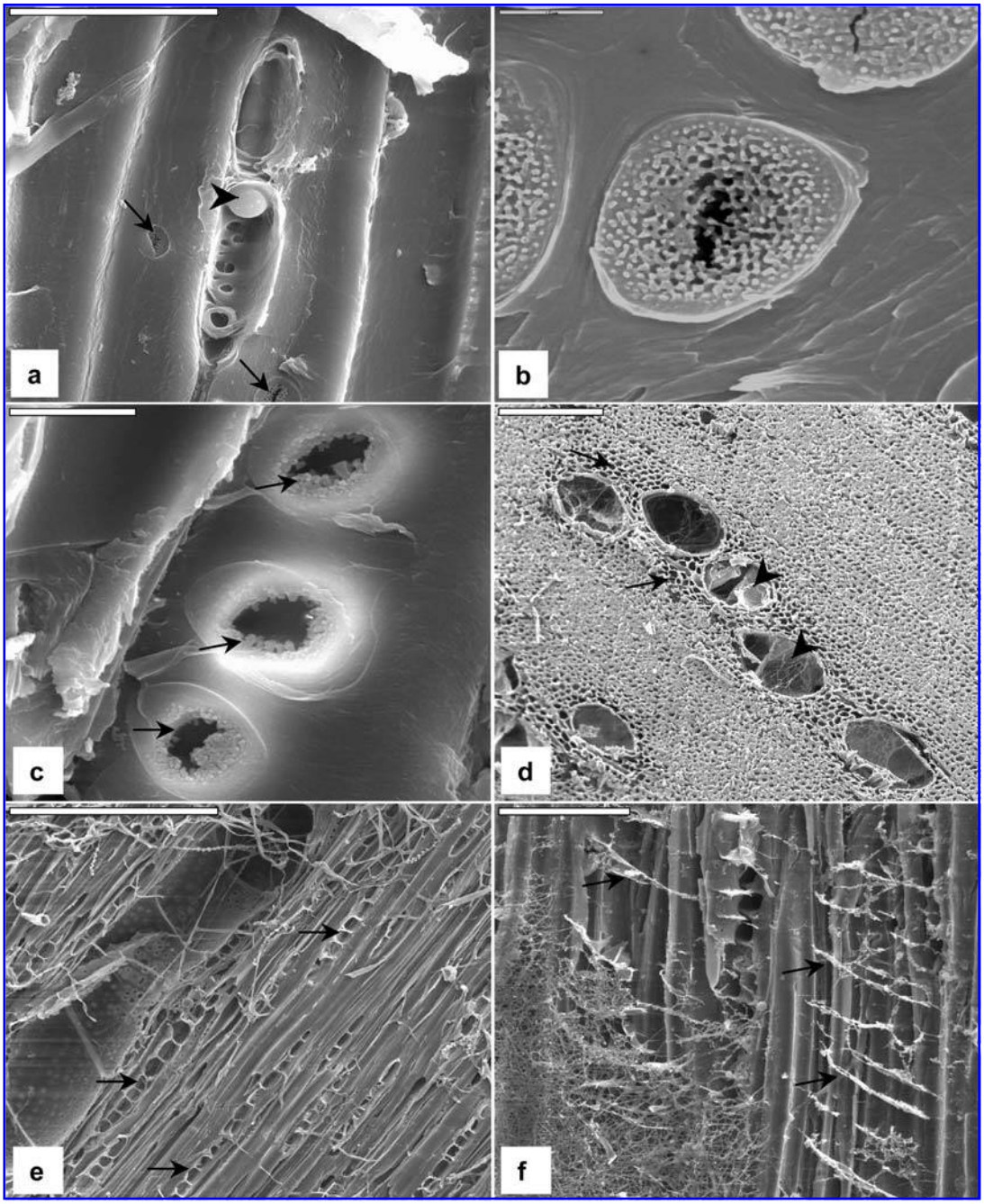

Figure 2 Low temperature microscopy images of *E. globulus* wood: (a–c) control and (d–f) treated with fungi for 14 days. (a) Spherical deposits in parenchyma rays (arrowheads) and bordered pits in fibers (arrows). (b) Intervessel pits showing vestures with protuberances. (c) Vestures protuberances removed from pit chamber after acetone extraction (arrows). (d) Tyloses (arrowheads) in vessels with hyphae and wood degraded areas (arrows) produced by *L. tigrinus*. (e) Absence of spherical deposits in parenchyma rays (arrows) in wood treated with *P. crassa*. (f) Abundant mycelium and degraded parenchyma cells (arrows) after wood treatment with *P. lycii*. Bars: (a) 20 μm, (b,c) 2 μm, (d,e) 200 μm, (h) 50 μm. (a–c,e) Tangential sections, (d) transverse section, (f) radial section.

of decay. In the case of wood treated with *P. lycii*, a strong loss of green autofluorescence was observed (Figure 3f), which is due to an extensive lignin degradation as determined by chemical analyses (Table 1). However, lignin located in vessel walls and fiber cell corners appeared to be more resistant to fungal attack (Figure 3f). Syringyl (S) units are known to be more easily biodegradable than the guaiacyl (G) lignin units. Lignin of *E. globulus* wood contains a lot of syringyl (S) units and thus the S/G ratio is around 2:1 (corrected value). It was found that *P. lycii* decreased the S/G ratio because of the preferential degradation of S units (del Río et al. 2002; Speranza 2003). As the vessel walls and fiber cell corners in *Eucalyptus* contain G units (Watanabe et al. 2004), this finding corroborates the statements above.

P. crassa and *P. lycii* also decreased heavily the yellow autofluorescence emitted from the cell lumen deposits. This observation can be interpreted as degradation of polyphenols, such as anthocyanidins, which were localized by specific staining in lumen deposits of *E. globulus* parenchyma (Hillis and Sumimoto 1989; Martínez et al. 1999).

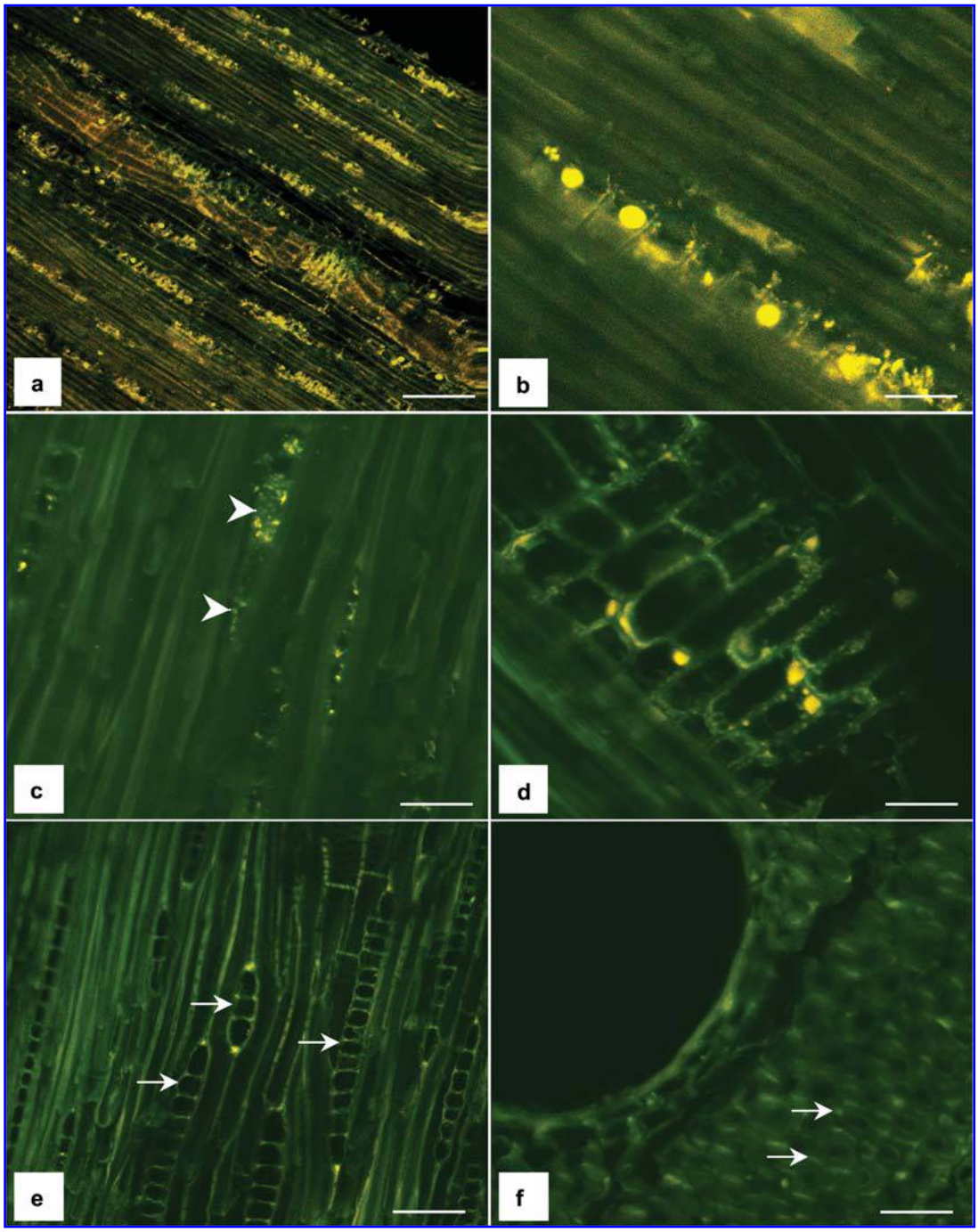

Figure 3 Confocal laser scanning microscopy images of *E. globulus* wood: (a–c) control and (d–f) treated with fungi for 14 days. (a) Autofluorescence of polyphenols (in rays), lignin, and cellulose. (b) Deposits with strong autofluorescence in parenchyma rays. (c) Small deposits (arrowheads) in parenchyma rays. (d) Autofluorescence in spherical deposits and cell walls after wood treatment with *L. tigrinus*. (e) Parenchyma ray (arrows) free of deposits in wood treated with *P. crassa*. (f) Autofluorescence remaining only in fiber cell corners (arrows) of wood treated with *P. lycii*. Image (a) is a combination of green and red channel color images; images (b–f) are collected in green channel. Bars: (a) 100 μm, (b,e) 10 μm, (c,d,f) 20 μm. (a,b,d) Radial sections, (c,e) tangential sections, (f) transversal section.

The fluorescence of sterol-filipin complexes was observed by FM in vessels, tyloses, and ray parenchyma cells in the control wood (Figure 4a–c). We suggest that these cells are the principal source of problematic lipophilic compounds, as reported by Speranza et al. (2002). The strongest filipin fluorescence signals were localized in spherical deposits (Figure 4a,b), which also showed polyphenol autofluorescence detected by CLSM (Figure 3a–c). The pit regions of vessels and ray parenchyma cell walls (Figure 4a and c) also displayed strong filipin fluorescence, although poor signals were detected in fiber pits (Figure 4a).

Vestured pits are very resistant to chemical treatment and it is difficult to elucidate their chemical nature (Jansen et al. 1998; Sano and Nakada 1998). Recently, alkali-soluble polyphenols and polysaccharides were detected

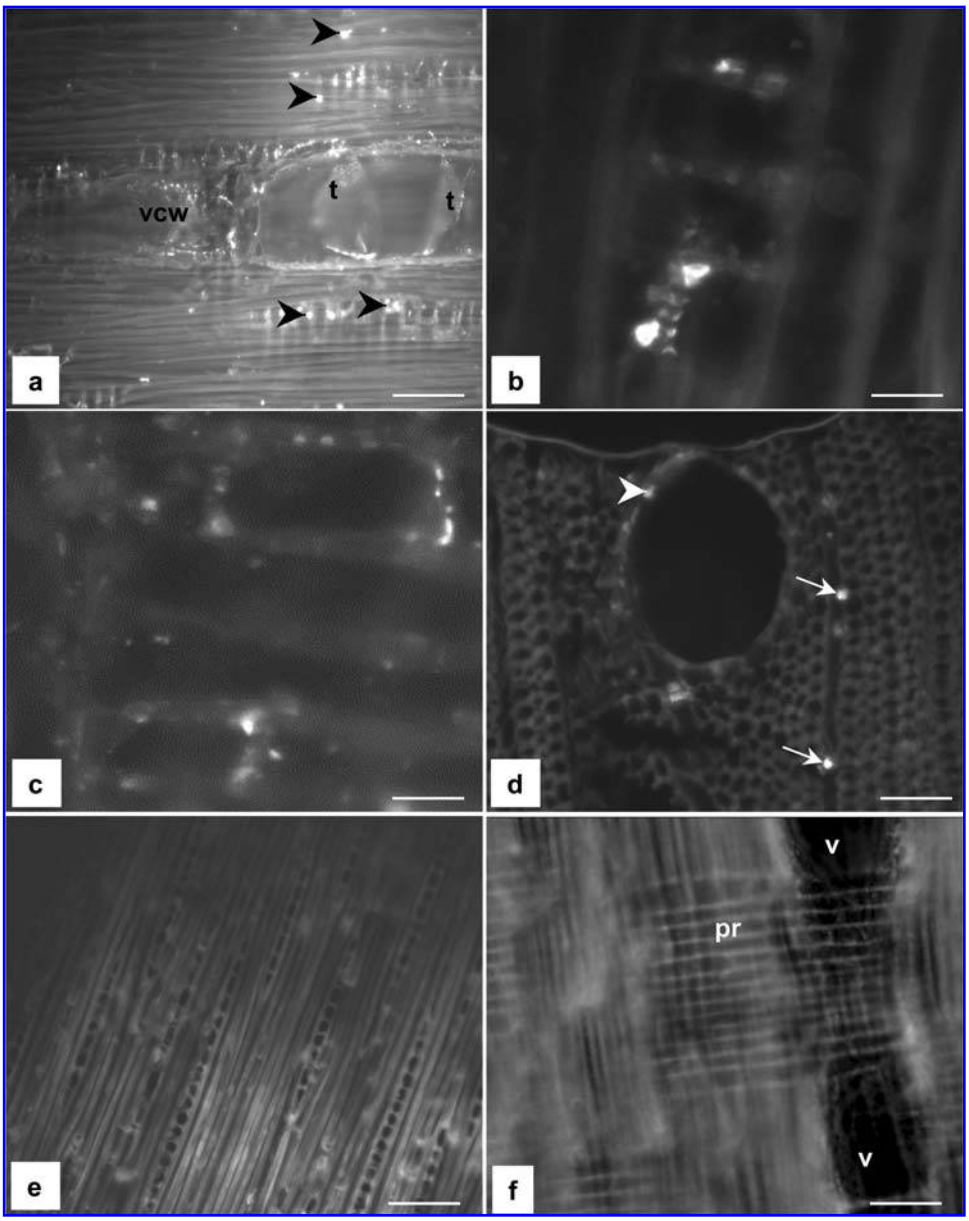

Figure 4 Images obtained by fluorescence microscopy of *E. globulus* wood stained with filipin: (a–c) control and (d–f) wood treated with fungi for 14 days. (a) Fluorescence produced by the sterols-filipin complexes in parenchyma rays deposits, vessel cell walls and tyloses. (b) Sterols-filipin fluorescence in deposits of parenchyma rays. (c) Fluorescence dots corresponding to the sterols-filipin complexes in simple pit chambers of parenchyma ray. (d) Sterols-filipin fluorescence in spherical deposits of parenchyma rays and in vessel wall remaining after wood treatment with *L. tigrinus*. (e,f) Absence of fluorescence complexes due to sterol degradation by *P. crassa* and *P. lycii*, respectively. Bars: (a,d–f) 100 μm, (b,c) 10 μm.

in vestured pits of *E. globulus* and *E. camaldunensis* by UV microscopy (Watanabe et al. 2006). In our experiments, free sterols were observed in vessel and parenchyma wood cells (Figure 4a–c), i.e., in zones that correspond to the vestured pits observed by LTSEM (Figure 2b). No filipin-sterol complexes were detected (data not shown) when the control was Soxhlet-extracted with acetone and the vestures were removed (Figure 2c).

In general, wood incubated with *L. tigrinus* A768 showed signals of filipin-sterol complexes present in spherical deposits of parenchymatic rays and in vessel cell walls (Figure 4d). These were similar to those observed in control wood (Figure 4a–c). However, no fluorescence signals were detected in most of the tyloses observed. In some zones of the wood decayed by *P. crassa*, mainly in parenchymatic rays, the filipin-sterol fluorescence disappeared (Figure 4e). This is in accordance with the observations made by LTSEM (Figure 2e) and CLSM (Figure 3e). *P. lycii* also caused a loss of filipin-sterol complexes fluorescence (Figure 4f). This is due to the extensive colonization and degradation of parenchyma rays observed by LTSEM (Figure 2f).

Conclusions

Filipin staining proved to be a simple and reliable method for determining the precise distribution of sterols and

allowed the detection of fungal degradation activity without artifacts (no pre-treatment of wood sections is necessary).

E. globulus wood contains sterols and polyphenols in the spherical deposits of ray parenchyma cells. Vessels also contain sterols. These cell types are the principal sources of problematic lipophilic compounds involved in pitch production. Sterols are also present in the vestures of fiber and vessel pits.

All the tested strains could degrade lignin, but only *P. lycii* showed a high specificity for lignin degradation within a short incubation period. However, the eucalypt lignin located in vessel walls and fiber cell corners was more resistant to fungal attack as revealed by CLSM. Parenchymatic rays and their lumen deposits were strongly degraded by *P. lycii* in accordance with the considerable sterol removal determined. *L. tigrinus* degraded sterols mainly in vessels and tyloses. *P. lycii* merit special attention concerning its high potential for biopulping and biodepitching processes.

Acknowledgements

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References

Akhtar, M., Blanchette, R.A., Myers, G., Kirk, T.K. (1998) An overview on biomechanical pulping research. In: Environmentally Friendly Technologies for the Pulp and Paper Industry. Eds. R.A. Young, M. Akhtar. TAPPI Press, Atlanta, GA. pp. 309–340.

Back, E.L., Allen, L.H. Pitch Control, Wood Resin and Deresination. TAPPI Press, Atlanta, GA, 2000.

Barsberg, S., Elder, T., Felby, C. (2003) Lignin-quinone interactions: implications for optical properties of lignin. Chem. Mater. 15:649–655.

Bettucci, L., Speranza, M., Siñeriz, F., Breccia, J.D. (1998) Degradation of *Eucalyptus globulus* and *Eucalyptus grandis* by several white rot fungi isolated in South America. Mater. Organismen ##:227–233.

Blanchette, R.A. (1995) Degradation of the lignocellulose complex in wood. Can. J. Bot. 73:S999–S1010.

Boyle, D. (1998) Nutritional factors limiting the growth of *Lentinula edodes* and other white-rot fungi in wood. Soil Biol. Biochem. 30:817–823.

Daniel, G. (1994) Use of electron microscopy for aiding our understanding of wood biodegradation. FEMS Microbiol. Rev. 13:199–233.

del Río, J.C., Speranza, M., Gutiérrez, A., Martínez, M.J., Martínez, A.T. (2002) Lignin attack during eucalypt wood decay by selected basidiomycetes: a Py-GC/MS study. J. Anal. Appl. Pyrolysis 64:421–431.

Donaldson, L., Hague, J., Snell, R. (2001) Lignin distribution in coppice poplar, linseed and wheat straw. Holzforschung 55:379–385.

Effland, M.J. (1977) Modified procedure to determine acid-insoluble lignin in wood and pulp. Tappi 60:143–144.

Farrell, R.L., Blanchette, R.A., Brush, T.S., Hadar, Y., Iverson, S., Krisa, K., Wendler, P.A., Zimmerman, W. (1993) Cartapip™: A biopulping product for control of pitch and resin acid problems in pulp mills. J. Biotechnol. 30:115–122.

Fernando, D., Daniel, G., Liden, J., Kraftliner, K. (2005) The state and spatial distribution of extractives during birch kraft pulping, as evaluated by staining techniques. Nordic Pulp Paper Res. J. 20:383–391.

Ferraz, A., Esposito, E., Bruns, R.E., Durán, N. (1998) The use of principal component analysis (PCA) for pattern recognition in *Eucalyptus grandis* wood biodegradation experiments. World J. Microbiol. Biotechnol. 14:487–490.

Ferraz, A., Parra, C., Freer, J., Baeza, J., Rodríguez, J. (2000) Characterization of white zones produced on *Pinus radiata* wood chips by *Ganoderma australe* and *Ceriporiopsis subvermispora*. World J. Microbiol. Biotechnol. 16:641–645.

Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., Evtuguin, D.V. (2006) Effect of oxygen, ozone and hydrogen peroxide bleaching stages on the contents and composition of extractives of *Eucalyptus globulus* kraft pulps. Bioresour. Technol. 97:420–428.

Griffith, G.S., Boddy, L. (1991) Fungal decomposition of attached angiosperm twigs. III. Effect of water potential and temperature on fungal growth, survival and decay of wood. New Phytol. 117:259–269.

Guerra, A., Mendonça, R., Ferraz, A., Lu, F.C., Ralph, J. (2004) Structural characterization of lignin during *Pinus taeda* wood treatment with *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. 70:4073–4078.

Gutiérrez, A., del Río, J.C., González-Vila, F.J., Martín, F. (1998) Analysis of lipophilic extractives from wood and pitch deposits by solid-phase extraction and gas chromatography. J. Chromatogr. 823:449–455.

Gutiérrez, A., del Río, J.C., Martínez, M.J., Martínez, A.T. (1999) Fungal degradation of lipophilic extractives in *Eucalyptus globulus* wood. Appl. Environ. Microbiol. 65:1367–1371.

Gutiérrez, A., del Río, J.C., Ibarra, D., Rencoret, J., Romero, J., Speranza, M., Camarero, S., Martínez, M.J., Martínez, A.T. (2006) Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. Environ. Sci. Technol. 40:3416–3422.

Hillis, W.E. Heartwood and Tree Exudates. Springer-Verlag, Berlin, 1987.

Hillis, W.E., Sumimoto, M. (1989) Effect of extractives on pulping. In: Natural Products of Woody Plants. II. Ed. J.W. Rowe. Springer-Verlag, Berlin. pp. 880–920.

Hunt, C., Kenealy, W., Horn, E., Houtman, C. (2004) A biopulping mechanism: Creation of acid groups on fiber. Holzforschung 58:434–439.

Jansen, S., Smets, E., Baas, P. (1998) Vestures in woody plants: a review. IAWA J. 19:347–382.

Martínez, M.J., Barrasa, J.M., Gutiérrez, A., del Río, J.C., Martínez, A.T. (1999) Fungal screening for biological removal of extractives from *Eucalyptus globulus* Labill. wood. Can. J. Bot. 77:1513–1522.

Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A., del Río, J.C. (2005) Biodegradation of lignocellulosics: Microbiological, chemical and enzymatic aspects of fungal attack to lignin. Int. Microbiol. 8:195–204.

Mendonca, R., Ferraz, A., Kordsachia, O., Patt, R. (2004) Alkaline sulfite/anthraquinone pulping of pine wood chips biotreated with *Ceriporiopsis subvermispora*. J. Chem. Technol. Biotechnol. 79:584–589.

Rayner, A.D.M., Boddy, L. Fungal Decomposition of Wood: Its Biology and Ecology. Wiley-Interscience Publishers, Chichester, 1988.

Sano, Y. (2005) Inter- and intraspecific structural variations among intervascular pit membranes, as revealed by field mission scanning electron microscopy. Am. J. Bot. 92:1077–1084.

Sano, Y., Nakada, R. (1998) Time course of the secondary deposition of incrusting materials on bordered pit membranes in *Cryptomeria japonica*. IAWA J. 19:285–299.

Scurfield, G., Silva, S.R. (1970) The vestured pits of *Eucalyptus regnans* F. Muell.: a study using scanning electron microscopy. Bot. J. Linn. Soc. 63:313–320.

Speranza, M. (2003) Hongos De La Podredumbre Blanca: Deslignificación y Control Biológico Del *Pitch* En *Eucalyptus Globulus*. PhD, Universidad de la República-PEDECIBA (Programa de Desarrollo de las Ciencias Básicas-PNUD), Montevideo.

Speranza, M., Martínez, M.J., Gutiérrez, A., del Río, J.C., Martínez, A.T. (2002) Wood and pulp localization of sterols involved in pitch deposition using filipin fluorescent staining. J. Pulp Paper Sci. 28:292–297.

Speranza, M., Ibarra, D., Romero, J., Martinez, A.T., Martínez, M.J., Camarero, S. (2007) Microscopy studies reveal delignification and sterols removal of eucalypt kraft pulps by laccase-HBT. Biocatal. Biotransform. 25:251–259.

Swan, B., Åkerblom, I.S. (1967) Wood extractives from *Eucalyptus globulus* Labill. Sven. Papperstidn. 70:239–244.

Tappi (1996–1997). Tappi Test Methods. TAPPI, Atlanta, GA, 1996.

Watanabe, Y., Kojima, Y., Ona, T., Asada, T., Sanol, Y., Fukazawa, K., Funada, R. (2004) Histochemical study on heterogeneity of lignin in *Eucalyptus* species II. The distribution of lignins and polyphenols in the walls of various cell types. IAWA J. 25:283–295.

Watanabe, Y., Sano, Y., Asada, T., Funada, R. (2006) Histochemical study of the chemical composition of vestured pits in two species of *Eucalyptus*. IAWA J. 27:33–43.

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