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Ability of selected wood-inhabiting fungi to degrade *in vitro* sapwood and heartwood of *Nothofagus pumilio*

Evaluación de la habilidad de algunos hongos xilófagos para degradar *in vitro* albura y duramen de *Nothofagus pumilio*

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ABSTRACT

Fungi are the main decomposers of lignocellulose in temperate forests and are classified as either white- or brown-rot, based on the ability to degrade lignin along with cellulose and hemicellulose. In this work, the decomposition of *Nothofagus pumilio* wood by different wood-inhabiting fungal species was investigated through *in vitro* assays. Sapwood and heartwood blocks were individually exposed to 11 fungal species; the dry mass loss was determined after 75, 135, and 195 days of exposure, comparatively analyzing the fungal ability to colonize and degrade this lignocellulosic substrate corresponding to both parts of the wood. Transverse sections of the blocks were made and separately stained with two types of dyes, Congo red and phloroglucinol, that are specifically associated with cellulose and lignin, respectively. Most of the species showed a different performance in sapwood and heartwood. *Rhizochaete brunnea*, *Aurantiporus albidus* and *Phanerochaete velutina* produced the greatest mass losses in the sapwood. The last two and *Laetiporus portentosus* produced the highest dry mass losses in heartwood, while *Rh. brunnea* was among the worst decomposers of this substrate. White rots generally showed a higher ability to degrade the

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sapwood and brown rotters the heartwood. The fungal species producing greater dry mass losses in heartwood than in sapwood grow on heartwood of living trees. Among white-rot fungi, two modes of action were identified: a) localized degradation, with zones of advanced decay in a less deteriorated matrix, and b) homogeneous degradation, with uniform decay. Our results showed that many species have different performances in different substrates, reinforcing the importance of analyzing sapwood and heartwood decomposition separately, something not usually done in this kind of studies.

Keywords — Brown-rot; light and fluorescent microscopy; mass loss; white-rot.

RESUMEN

Los hongos son los principales descomponedores lignocelulolíticos de los bosques templados, y se clasifican en pudridores blancos o castaños, según puedan o no degradar la lignina junto con la celulosa y la hemicelulosa. En este trabajo, la descomposición de la madera de *Nothofagus pumilio* por parte de diferentes especies fúngicas fue investigada mediante ensayos *in vitro*. Bloques de albura y de duramen fueron individualmente expuestos a 11 especies fúngicas; la pérdida de peso seco fue determinada a los 75, 135 y 195 días, analizando comparativamente la habilidad de los hongos para colonizar y degradar este sustrato lignocelulósico correspondiente a ambos regiones del leño. Se realizaron cortes transversales de los bloques y se tiñeron separadamente con dos tipos de colorantes, rojo Congo y fluoroglucinol, que se asocian específicamente a celulosa y lignina, respectivamente. La mayoría de las especies mostraron un distinto desempeño en albura y en duramen. *Rhizochaete brunnea*, *Aurantiporus albidus* y *Phanerochaete velutina* produjeron las mayores pérdidas de peso seco en albura. Los dos últimos y *Laetiporus portentosus* produjeron las mayores pérdidas de peso seco en duramen, mientras que *Rh. brunnea* estuvo dentro de los peores descomponedores de este sustrato. En general, los pudridores blancos mostraron una mayor habilidad para degradar la albura, mientras que los pudridores marrones fueron mejores en duramen. Todas las especies que produjeron mayores pérdidas de peso seco en duramen crecen en el duramen de árboles vivos. Dentro de los pudridores blancos, dos patrones de acción fueron identificados: a) localizado, con zonas con degradación avanzada dentro de una matriz menos deteriorada; b) homogéneo, con una degradación uniforme. Nuestros resultados muestran que muchas especies tienen diferente desempeño en distintos sustratos, reforzando la importancia de analizar separadamente la descomposición de albura y duramen, algo que no se hace usualmente en este tipo de trabajos.

Palabras clave — Microscopía óptica y de fluorescencia; pérdida de peso seco; pudridores blancos; pudridores marrones.

INTRODUCTION

Wood is colonized and degraded by a wide variety of biological agents, being the Basidiomycetes the main decomposers in forests (Rayner & Boddy, 1988a). These wood decay fungi have typically been classified as either white-rot or brown-rot, whether or not they can degrade lignin along with cellulose and hemicellulose. Most of them are white-rot; brown-rot fungi constitute only 6% of the described wood decay fungal species. The first ones are predominantly associated with hardwoods, while the latter ones are with softwoods (Rayner & Boddy, 1988b; Blanchette, 1995).

White-rot is subdivided into simultaneous rot, where all the cell wall components are degraded at the same time, and selective delignification, where lignin is degraded earlier than holocellulose. This kind of decay can cause high mass losses, in comparison with brown-rot decay (Schwarze, 2007). Regarding this last one, it is known that brown-rot fungi cause a rapid loss in wood strength at a minimal mass loss. These species break down cellulose and hemicelluloses; also, they can cause an extensive modification of lignin via demethoxylation (Green & Highley, 1997). Although they cannot cause delignification, brown-rot fungi have a well-developed mechanism for overcoming the lignin barrier; evidence suggests that their decay system involves a two-phase attack in which the wood cell wall is initially opened up by a rapid effective oxidative low molecular weight system, which then provides conditions for an enzymatic system involving hydrolytic and/or oxidative enzyme systems (Daniel, 2016). Low molecular weight compounds play important roles in all stages of wood decay, acting as diffusible oxidative agents and electron shuttles for enzymatic systems. These fungal metabolites are important during the early stages of decay because fungal enzymes are too large to penetrate the intact wood cell wall (Janusz *et al.*, 2017).

Hardwood xylem is composed of a variety of cell types specialized in different functions. Vessels, the most characteristic cell type (Daniel, 2016), have water conducting functions, and fibers or fiber tracheids supply mechanical strength and support; xylem rays and axial parenchyma have parenchymatous cells for storage and nutrient and water transport between xylem and phloem (Schwarze, 2007). Although all wood cells are composed of different ratios of cellulose, lignin, and hemicelluloses, differences can exist in wood chemistry within different cell types, and even across wood cell walls and between the compound middle lamella separating wood cells (Saka *et al.*, 1982).

The wood cell wall is organized in layers of different thicknesses and different ratios of cellulose, hemicellulose, and the matrix material lignin. First, a thin primary wall is formed, to which a much thicker secondary wall, consisting of three layers (S_1, S_2, and S_3), is then added. Completion of secondary wall formation and the lignification of cell walls in fibers and vessels is followed by the death of the cells. Parenchyma cells, however, differ from the other cells in that they are living as long as they are part of the sapwood (Harada & Côté, 1985). Walls of adjacent cells are bonded together by the middle lamella (Wardrop & Harada, 1965). The highest lignin content is always found in the middle lamella while the S_2 layer contains the greatest total volume of lignin, cellulose, and hemicelluloses. These differences in

the micro-distribution of the different chemical components at the wood cell wall level can affect fungal decay, because the chemistry of wood cell walls has a major influence on how rapid cell walls are degraded by different fungi (Daniel, 2016). In particular, wooden plant cell walls have developed a very robust structural framework, where cellulose microfibrils are embedded in a lignin matrix (Boer *et al.*, 2005). This lignin, and hemicellulose polymers also present in the cell wall, function as a recalcitrant adhesive for cellulose (Janusz *et al.*, 2017). Moreover, lignin prevents access of low molecular weight diffusible agents, which are required for decomposition of cellulose and hemicelluloses (Schwarze, 2007). Hence, wood degrading fungi have to overcome this barrier and, although different species are able to decompose these polysaccharides, only a group of fungi can completely degrade lignin (Blanchette, 1995; Dashtban *et al.*, 2010).

Nothofagus pumilio (lenga) is the main native forest resource in Patagonia (Argentina), and its wood-inhabiting fungi have been thoroughly described (Cwielong & Rajchenberg, 1995; Rajchenberg, 1996; Greslebin, 2002; Rajchenberg, 2006). Five species account for 85% of the living tree rots: *Nothophellinus andinopatagonicus* (Hymenochaetaceae), *Aurantiporus albidus* (Meruliaceae), *Postia pelliculosa* (Dacryobolaceae), *Laetiporus portentosus* (Laetiporaceae), and *Serpula himantioides* (Serpulaceae). The first two are white-rot, while the last tree are brown-rot fungi (Cwielong & Rajchenberg, 1995; Rajchenberg, 1996). Besides, in fallen woody debris, over a hundred fungal species have been recorded (Greslebin, 2002; Rajchenberg, 2006), which can grow in different woody tissues and play specific roles in the decomposition process. *Phanerochaete velutina* (Phanerochaetaceae), *Xylodon raduloides* (Schizoporaceae), and *Stereum hirsutum* (Steraceae) are among the most abundant species in fallen wood. Other fungi, such as *Hymenochaete australis* (Hymenochaetaceae), *Stereodiscus triviale* (Stereaceae) and *Leptosporomyces luteofibrillosus* (Atheliaceae) have particular characteristics regarding the stage of decay where they develop. The first two are pioneers, while the last one is related to advanced decay stages and also to standing trees (Greslebin & Rajchenberg, 2003). Besides, other species grow exclusively in wood of particular diameters; *H. australis* in small branches (< 10 cm diameter) and *L. luteofibrillosus* in logs (> 20 cm diameter) (Greslebin & Rajchenberg, 2003). *Rhizochaete brunnea* (Phanerochaetaceae) belongs to the same clade that *Phanerochaete*, a genus that counts with well-known wood decaying species used in biotechnology (Greslebin *et al.*, 2004).

The objective of this study was to determine the ability of 11 wood-inhabiting fungal species, most of them endemic from the Patagonian *N. pumilio* forests, to decompose wood blocks of *N. pumilio* under a pure culture condition, and to analyze the structural and/or chemical changes in woody tissues and in the composition of the wall cells produced by each fungus, using histochemical techniques.

MATERIALS AND METHODS

Fungal strains

Fungal strains corresponding to eleven wood-inhabiting species were obtained from the culture collection of CIEFAP (*Aurantiporus albidus* CIEFAPcc 117, *Hymenochaete australis* CIEFAPcc 211, *Laetiporus portentosus* CIEFAPcc 132, *Leptosporomyces luteofibrillosus* CIEFAPcc 208, *Nothophellinus andinopatagonicus* CIEFAPcc 42, *Phanerochaete velutina* CIEFAPcc 613, *Postia pelliculosa* CIEFAPcc 179, *Rhizochaete brunnea* CIEFAPcc 229, *Stereodiscus triviale* CIEFAPcc 232, *Stereum hirsutum* CIEFAPcc 28, *Xylodon raduloides* CIEFAPcc 106). All strains had been obtained from fungi growing on *N. pumilio* wood, and are representative of the species to which they belong. The strains were cultured at 23°C on Petri dishes with MEA medium (malt extract 2%, agar 1.5%).

In vitro wood decomposition assay

To determine whether different fungal species cause different wood mass loss, and whether the same species has a different performance in sapwood and heartwood, an *in vitro* wood decomposition assay with 11 different species was conducted under pure culture conditions. Sapwood and heartwood blocks were cut from *N. pumilio* sound trees (2 x 2 x 1 cm^3), and oven-dried at 60°C until constant weight. Then, blocks were saturated with distilled water for 48 h and sterilized for 2 h, following Robles *et al.* (2011) with modifications. A 10-day-old culture of each strain was used as inoculum, and it was transferred by punching out a 10 mm surface agar plug to a Petri dish (90 mm in diameter) filled with sterile MEA medium. Petri dishes were incubated until, at least, 75% colonization of the surface medium by the mycelium was reached. Then, a sterilized block of sapwood or heartwood was placed on the center of the mycelia mat. In all cases, the side of the block lying on the mycelium was the cross section. Five replicates for each species in each substrate (sapwood and heartwood) were done; blocks in un-inoculated Petri dishes served as controls. Wood blocks were incubated at 23°C and 35% relative humidity. Mass loss was used as an estimator of the decay ability of each species. To determine mass loss over time, three harvesting times were established (75, 135, and 195 days). The blocks were retrieved, cleaned, oven-dried at 60°C until constant weight, and weighed. Mass loss was determined as a percentage of the original mass.

To evaluate and compare the decay ability of the different fungal species on the decomposition of the wood blocks, a linear mixed model was performed using the mean value of the percentage of mass loss of the wood in the three harvesting times analyzed. These mean values were further compared using Fisher's least significant difference (LSD) at p level <0.05. The factors were 'treatment' (with eleven levels corresponding to the different fungal species), 'time' (with three levels: 75, 135, and 195 days) and 'substrate' (with two levels: sapwood and heartwood); the factors were treated as fixed effects. Normality assumption was evaluated through Shapiro-Wilks tests, and homoscedasticity assumption through residuals. If heteroscedasticity was

detected, a second test modeling was carried out. Both models were compared and, if $p < 0.05$, the one with the lower Akaike (AIC) value was selected. To determine whether a species has a preference for sapwood or heartwood blocks, a second linear mixed model was performed using the same response variable (mean value of the percentage of mass loss). In this case, the factor was 'substrate' (with two levels: sapwood and heartwood) and it was treated as fixed effect. Significant interactions were observed between the factors 'treatment', 'substrate', and 'time'; hence, they were analyzed separately. All modeling and statistical analyses were performed in InfoStat software (Di Rienzo *et al.*, 2018). InfoStat implements an interface of the R platform (R Core Team, 2018) for estimation of linear mixed models through the functions 'gls' and 'lme' from Nonlinear Mixed-Effects Models library (Pinheiro & Bates, 2004; Di Rienzo *et al.*, 2018). To create the artworks, Infostat software and Corel DRAW X7 were used.

Histochemistry

To analyze differences between un-attacked wood and one attacked by each species studied, microscopic slides of the dried blocks (10 ìm thick) in transverse section were performed using Leica Hn 40 microtome and treated with two different staining methods: phloroglucinol (for staining lignin) (Mauch-Mani & Slusarenko, 1996), and congo red (for staining holocellulose) according to Mitra & Loqué (2014) with the following modifications: transverse sections were placed on a slide, covered with several drops of congo red 0.5%, and kept overnight at 4°C. Then, sections were rinsed with distilled water, until the washing solution was clear, and observed, within 60 min, under UV-light excitation using a filter with a bandpass of 340-380 nm. Other stains were tried (toluidine blue, fluorescent brightener 28, safranin-fast green) before choosing the ones shown here. Observations were made in a light microscope Leica DM500 with epifluorescence and photomicrographs were obtained by Canon EOS Rebel T3i digital camera. The images were processed on ImageJ 1.53.

RESULTS AND DISCUSSION

In vitro wood decomposition assay

After 195 days, mean mass loss caused by the different fungal species ranged from 3% to 16% in sapwood blocks, and from 3% to 9% in heartwood blocks. Significant differences in wood mass loss between fungal species, and also differences in the ability to decompose sapwood and heartwood were found (Fig. 1; Fig. 2).

Based on mass losses shown in Fig. 1, three groups of species could be delimited: those that produced the greatest mass losses (>10%), those that produced intermediate (>5% and <10%), and those that produced the lowest (<5%). The greatest mass losses in sapwood were caused by *Rh. brunnea*, *A. albidus* and *Ph. velutina*. *Aurantioporus albidus* is one of the white-rot fungi that colonize and decompose the stand living tree causing, together with five other species, up to 85% of *N. pumilio*

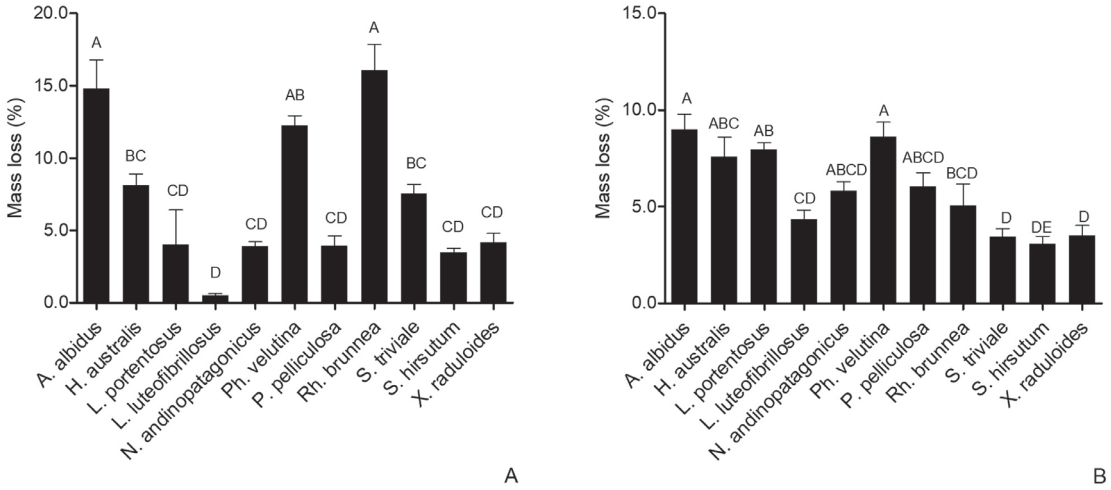

Fig. 1. Mean mass loss of sapwood (A) and heartwood (B) blocks caused by different wood-inhabiting fungal species after 195 days of incubation. Media ± SE are shown. n=5. Different letters indicate significant differences ($p < 0.05$).

Fig. 1. Pérdida de masa promedio de los bloques de albura (A) y duramen (B) causada por diferentes especies de hongos de la madera después de 195 días de incubación. Se muestran las medias ± EE. n=5. Letras diferentes indican diferencias significativas ($p < 0,05$).

living rots (Cwielong & Rajchenberg, 1995), and *Ph. velutina* and *Rh. brunnea* are two phylogenetically related species that grow on fallen dead wood (Greslebin *et al.*, 2004). The second group of fungi, formed by *H. australis* and *S. triviale*, corresponds to saprotrophic species that mostly grow on fallen wood, although *H. australis* can also be found in dead branches of standing trees (Greslebin & Rajchenberg, 2003). All the other species caused sapwood mean mass losses of only up to 5% or less (Fig. 1). Similarly, in heartwood, the greatest mass losses were caused by *A. albidus* and by *Ph. velutina*, but to a lesser extent than those they caused in sapwood (Fig. 1; Figs. 2A, 2F). The second group was composed, again, by *H. australis*, but also by *L. portentosus*, *P. pelliculosa* y *N. andinopatagonicus* which were in the lowest mass loss group in sapwood. A distinction between species regarding their performance in the different substrates is evidenced here since, contrary to most of the other species, these three species showed higher mass losses in heartwood than in sapwood (Fig. 1; Figs. 2C, 2E, 2G), which were significant in the case of *L. portentosus* and *N. andinopatagonicus*. These tree species constitute, together with *A. albidus*, the main species causing heart-rot of standing *N. pumilio* trees (Cwielong & Rajchenberg, 1995). *Rhizochaete brunnea,* the species which caused the greatest mass loss in sapwood, was in the group of species that caused the lower mass losses in heartwood, which reinforces the differences in species' performance according to the substrate nature.

In the tree, the sapwood is the part of the wood in which some xylem cells are still alive; hence is physiologically active, contrary to the heartwood, which is the physiologically dead part of the xylem (Harada & Cote, 1985). This can be the reason why only a few species of fungi can develop in the sapwood of a living tree but, once the tree is dead, this substrate is accessible and offers more nutrients than heartwood which is also more recalcitrant due to the higher concentration of extractives. Our *in vitro* assays showed that sixty-four percent of the tested species showed differential

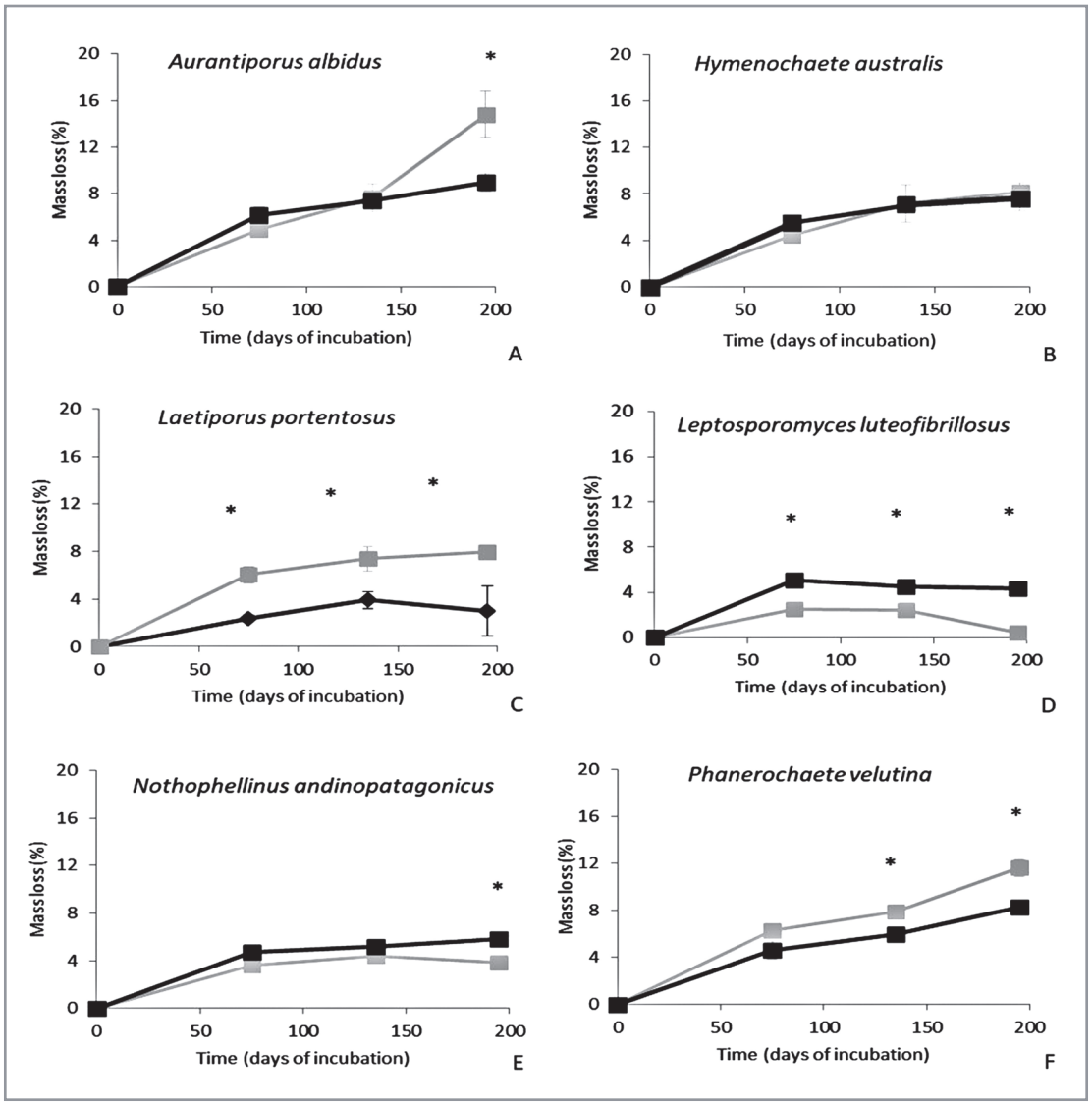

Fig. 2 (part 1 of 2). Mean mass loss of sapwood (gray) and heartwood (black) blocks caused by different wood-inhabiting fungal species after 75, 135, and 195 days of incubation. A) *Aurantiporus albidus* B) *Hymenochaete australis* C) *Laetiporus portentosus* D) *Leptosporomyces luteofibrillosus* E) *Nothophellinus andinopatagonicus* F) *Phanerochaete velutina* G) *Postia pelliculosa* H) *Rhizochaete brunnea* I) *Stereodiscus triviale* J) *Stereum hirsutum* K) *Xylodon raduloides*. Media ± SE are shown. Significant differences are indicated with * ($p < 0.05$). n=5.

Fig. 2 (parte 1 de 2). Pérdida de masa promedio de bloques de albura (gris) y duramen (negro) causada por diferentes especies de hongos de la madera después de 75, 135 y 195 días de incubación. A) *Aurantiporus albidus* B) *Hymenochaete australis* C) *Laetiporus portentosus* D) *Leptosporomyces luteofibrillosus* E) *Nothophellinus andinopatagonicus* F) *Phanerochaete velutina* G) *Postia pelliculosa* H) *Rhizochaete brunnea* I) *Stereodiscus triviale* J) *Stereum hirsutum* K) *Xylodon raduloides*. Se muestran las medias ± EE. Las diferencias significativas se indican con * ($p < 0,05$). n=5.

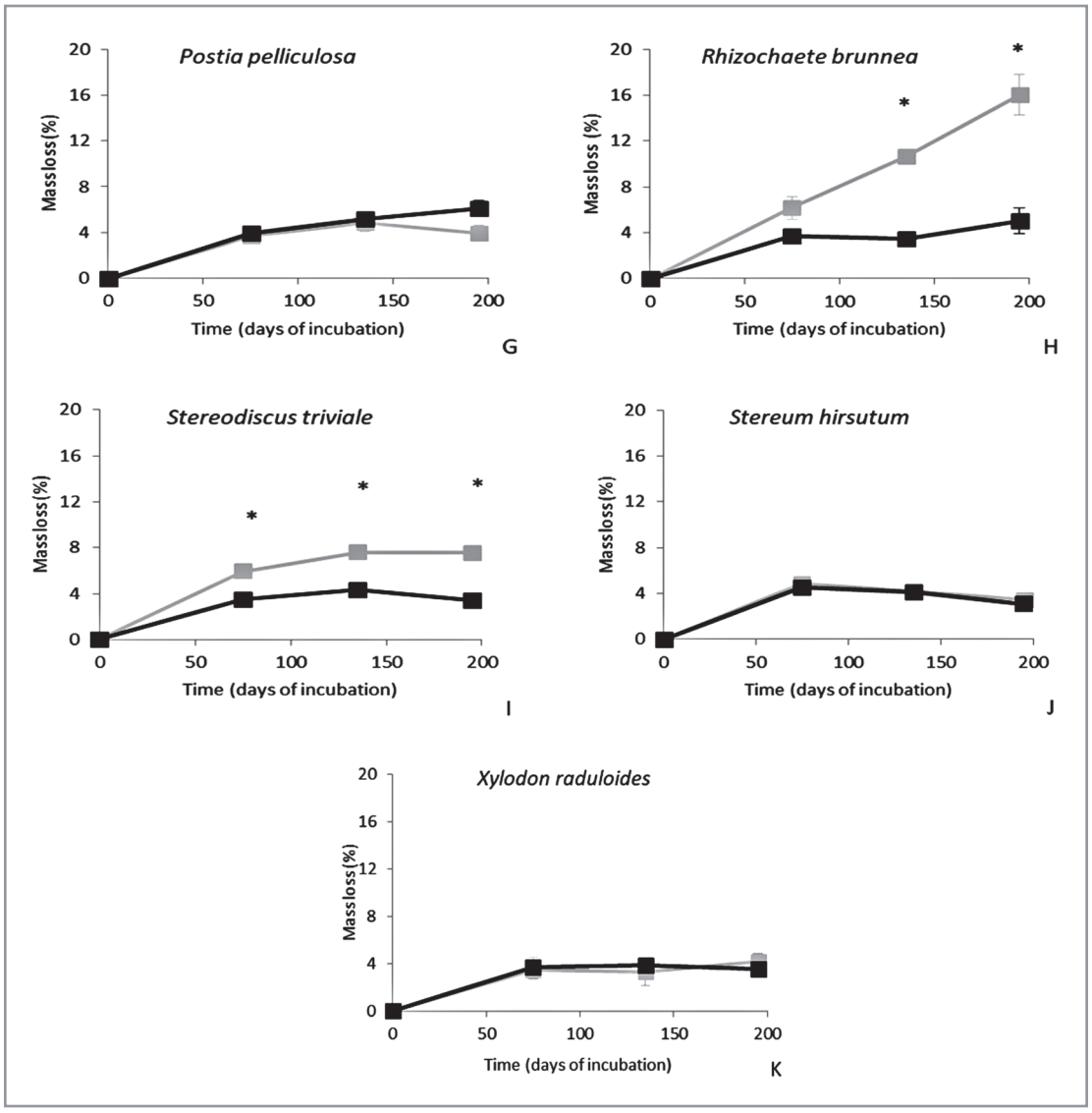

Fig. 2 (part 2 of 2).

Fig. 2 (parte 2 de 2).

ability to decompose these different parts of the tree (Fig. 2). Most of them caused higher mass losses in sapwood than in heartwood blocks, except for *L. portentosus*, *L. luteofibrillosus*, *P. pelliculosa* -the 3 brown-rot fungi included in the study-, and *N. andinopatagonicus* -the main white heart-rot species of *N. pumilio* (Rajchenberg, 2006)-, which showed the opposite pattern (Figs. 2 D-F, 2H). Sapwood is a tissue with many defense compounds in the living tree but, once this tissue is dead, it is an easier material to decompose because the relative amount and nature (aromaticity) of its lignin is lesser than that in heartwood (Boddy, 2001). Also, heartwood has more extractives than sapwood, and it has been shown that these compounds do not favor *in vitro* decomposition of woody blocks of other plant species (Mohareb *et al.*, 2010), probably because they might be fungitoxic compounds, which are deposited in the heartwood and prevent decay (Schwarze, 2007). These could explain why most species caused a higher mass loss in sapwood than in heartwood.

Regarding those species which caused a higher mass loss in heartwood than in sapwood, all of them are species that grow in the heartwood of living trees (Rajchenberg, 2006), a feature that might be associated with their better performance in this part of the tree. *Laetiporus portentosus* turned out to be one of the best heartwood decomposers and one of the worst sapwood decomposers. Moreover, as mentioned above, *N. andinopataganicus* and *L. luteofibrillosus*, along with *P. pelliculosa* and *A. albidus*, are the main heartwood rots of standing *N. pumilio* trees, which could indicate that they have a selective advantage or some type of specialization for this part of the tree. Besides, although *L. luteofibrillosus* produced low mass losses in both substrates, the better performance in heartwood agrees with its almost exclusive presence in much-decayed heartwood of standing or fallen trees (Rajchenberg, 1996; Greslebin & Rajchenberg, 2003).

Over time, a different performance among species was found. Some fungi caused mass loss until the end of the assay: *Ph. velutina* in both substrates (Fig. 2F), and *A. albidus* and *Rh. brunnea* in sapwood (Figs. 2A, 2H). Meanwhile, most of the tested species caused a higher mass loss during the first 75 days, and this was more evident in heartwood than in sapwood (Fig. 2). In accordance with our results, Song *et al.* (2010) found that *in vitro* mass loss is higher in the first five weeks than in the last five.

Histochemistry revealed some aspects of the interactions between wood structure and fungal activity. Microscopic examinations of wood samples obtained from blocks that were incubated for 195 days with the different fungal species showed that the way in which decomposition took place varied among different fungal species, which is consistent with the different types of decay. For histochemical analysis we only considered the first two groups of species: those that caused the greatest mass losses (*A. albidus*, *Rh. brunnea,* and *Ph. velutina*), and those that caused intermediate mass losses (*S. triviale*, *H. australis,* and *L. portentosus*). The appearance of transverse sections of uninoculated control blocks is presented in Fig. 3.

The three species that caused the greatest mass losses in sapwood were all white-rot fungi, but their modes of action were different. In the blocks inoculated with *A. albidus* or *Rh. brunnea*, areas with different degrees of delignification were observed; this seems to be a localized process with zones of advanced decay close to a less deteriorated zone or in a less decayed matrix, as it can be observed in Figs. 4 A-C, where fibers with a very advanced decay (white arrows) are next to fibers with little decay (blue arrows). The loss of lignin was evidenced by the lesser fluorescence (which corresponds to the autofluorescence of lignin in UV light) with respect to the control. Lignin seems to have been firstly removed from the secondary wall layers S_2 and S_3 near the lumen and, then, throughout the S_2 toward the S_1 and the middle lamella with the consequent cell wall thinning and boreholes (Figs. 4A, black arrows; 4C, white arrows), and finally, walls were broken (Fig. 4A, white arrows). The middle lamella and cell corners were the least degraded areas (Figs. 4A, 4C). On the contrary, in the blocks inoculated with *Ph. velutina* a generalized delignification (evidenced by the diminution of autofluorescence) of fiber cell walls, including the middle lamella, was observed. Some fluorescence was only found in the corners, which can be attributed to their pronounced lignification (Fergus & Goring, 1970); no boreholes nor thinner walls in specific zones were found (Fig. 4E). In some fibers, a G layer

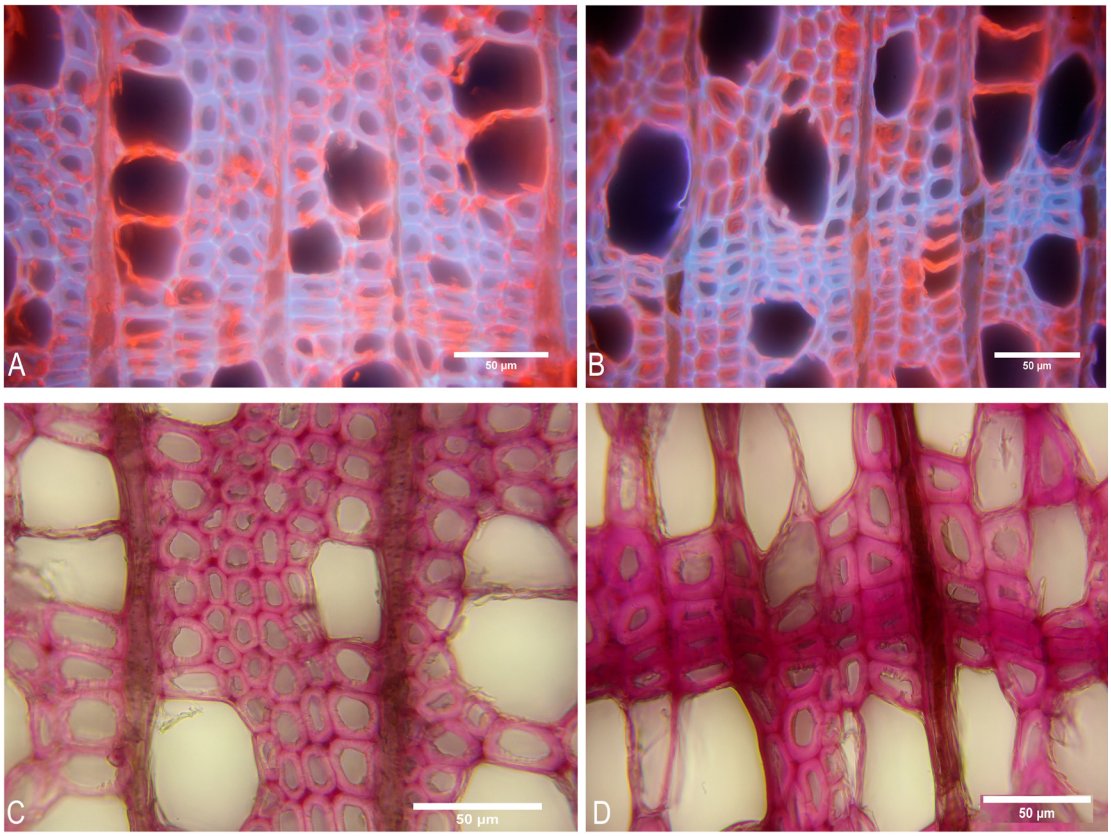

Fig. 3. Microscopic transverse sections of uninoculated control blocks: A) and B) Sapwood and heartwood, respectively, treated with Congo red stain (overnight) and photographed under UV light. An intense white fluorescence is observed in both slices indicating the presence of an un-altered matrix of lignin masking holocellulose. Vessel walls show red color indicating that cellulose is exposed in these cells, which binds Congo red dye. C) and D) Sapwood and heartwood, respectively, treated with phloroglucinol. Both slices show an intense pink color indicating the lignin deposition in cell walls.

Fig. 3. Cortes transversales microscópicos de bloques control sin inocular: A) y B) Albura y duramen respectivamente, tratados con colorante rojo Congo (durante una noche) y fotografiados bajo luz ultravioleta. Se observa una intensa fluorescencia blanca en ambos cortes que indica la presencia de una matriz inalterada de lignina que enmascara la holocelulosa. Las paredes de los vasos muestran un color rojo que indica que la celulosa está expuesta en estas células, y que se une al colorante rojo Congo. C) y D) Albura y duramen respectivamente, tratados con floroglucinol. Ambos cortes muestran un color rosa intenso que indica el depósito de lignina en las paredes celulares.

was detected. It seems that, in blocks inoculated with *Ph. velutina*, the delignification affects the fiber cell wall homogeneously. In heartwood blocks, even though mass losses caused by these species were lower than in sapwood, the pattern was similar. Delignification in blocks inoculated with *A. albidus* and *Rh. brunnea* was evidenced by the loss of fluorescence, although the marked thinning observed in the fiber cell walls in sapwood was not detected in this case (Figs. 4B, 4D). *Phanerochaete velutina* also caused an homogenous delignification in heartwood, but the middle lamellae still fluoreced intensely, showing less lignin loss than in sapwood (Fig. 4F).

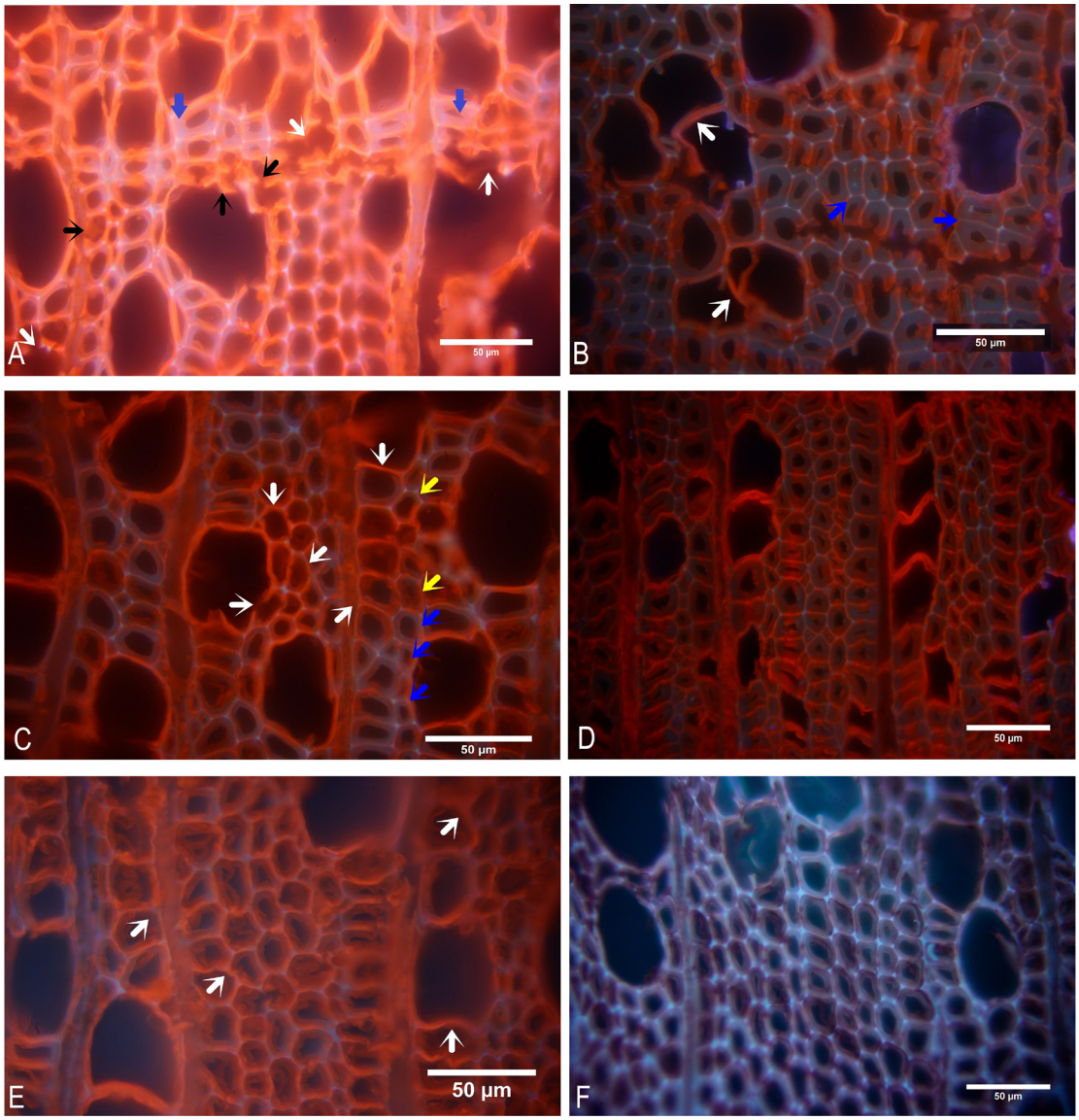

Fig. 4. Microscopic transverse sections of blocks treated with Congo red stain and photographed under UV light, and inoculated with: *Aurantiporus albidus* (A-B), *Rhizochaete brunnea* (C-D) and *Phanerochaete velutina* (E-F). A) Sapwood: zones with great delignification show the red color of fiber walls and the loss of white fluorescence, thinned fiber walls (black arrows), and much degraded broken walls (white arrows). Some fibers show less degradation evidenced by the blue color of fiber walls and the white fluorescence of the middle lamella (blue arrows). B) Heartwood: the lower fluorescence than the control shows delignification but to a lesser extent than sapwood, fiber walls are not thinned, and middle lamella shows white fluorescence (blue arrows), vessel walls show red color and thinning (white arrows). C) Sapwood: fiber cell walls in a very decayed (white arrows), with intermediate decay (yellow arrows), and with little decay (blue arrows). White arrows point out areas of the fibers wall that have been almost totally degraded and only middle lamella remains. D) Heartwood: fiber walls are not thinned but the decrease of white fluorescence and structure shows delignification, to a lesser extent than sapwood. E) Sapwood: Homogeneous loss of lignin, evidenced by the red color, the loss of white fluorescence, and the thinned fiber walls (white arrows). F) Heartwood: homogeneous delignification of fiber walls (dark red color), but not in the middle lamella which still has a white fluorescence.

Fig. 4. Cortes transversales microscópicos de bloques tratados con colorante rojo Congo y fotografiados bajo luz ultravioleta e inoculados con*: Aurantiporus albidus* (A-B), *Rhizochaete brunnea* (C-D) y *Phanerochaete velutina* (E-F). A) Albura: las zonas con gran delignificación muestran coloración roja de las paredes de las fibras y pérdida de la fluorescencia blanca, paredes adelgazadas de las fibras (flechas negras) y paredes rotas muy degradadas (flechas blancas). Algunas fibras muestran menos

degradación evidenciada por el color azul de las paredes de la fibra y la fluorescencia blanca de la laminilla media (flecha azul). B) Duramen: la menor fluorescencia, en comparación con el control, muestra delignificación pero en menor medida que la albura, las paredes de las fibras no están adelgazadas y la laminilla media muestra fluorescencia blanca (flechas azules), las paredes de los vasos muestran coloración roja y adelgazamiento (flechas blancas). C) Albura: áreas del tejido muy degradadas (flechas blancas), con degradación intermedia (flechas amarillas) y poco degradadas (flechas azules). Las flechas blancas señalan áreas de la pared de las fibras que se han degradado casi por completo y donde sólo queda la laminilla media. D) Duramen: las paredes de las fibras no están adelgazadas, pero la disminución de la fluorescencia blanca y la estructura evidencian delignificación, aunque en menor medida que en la albura. E) Albura: Pérdida homogénea de lignina, evidenciada por el color rojo, la pérdida de fluorescencia blanca y el adelgazamiento de las paredes de las fibras (flechas blancas). F) Duramen: delignificación homogénea de las paredes de la fibra (color rojo oscuro), pero no de la laminilla media que aún presenta una fluorescencia blanca.

The second group of species, in terms of mass loss, comprised three fungi: two of them described as white-rot (*S. triviale* and *H. australis*) and one as brown-rot (*L. portentosus*). The performance of each species in sapwood and in heartwood was different. *S. triviale* caused a significantly higher mass loss of sapwood blocks than of heartwood blocks; the opposite was found for *L. portentosus*. Meanwhile, mass losses of sapwood and heartwood blocks inoculated with *H. australis* did not differ significantly. The two white-rot species showed an uneven decay pattern, as observed in *A. albidus* and *Rh. brunnea*, with some areas more degraded than others (Figs. 5 A-D, white and blue arrows respectively). Sapwood blocks inoculated with *S. triviale* showed, when stained with the congo red dye, some loss of the structure of vessels and fibers, evidenced by the alteration of their shape (Fig. 5A, white and yellow arrows). The decrease in fiber cell walls fluorescence and the red stain in some areas evidenced the loss of lignin. Delignification of fibers seems to start in the inner layer (Fig. 5A, yellow arrow), mostly affecting S_2. The middle lamella and cell corners still showed fluorescence, indicating the presence of lignin. Heartwood blocks revealed fewer degradation signs than sapwood blocks, evidenced by their greater fluorescence, the lesser red color, and the maintenance of the structure of both fiber and vessel walls (Fig. 5B), although wall thinning was observed in some of these cells (white and yellow arrows respectively). In the case of blocks inoculated with *H. australis*, both sapwood and heartwood showed a similar level of degradation and the same pattern when stained with congo red: the early wood was more attacked than the latewood (Figs. 5C, 5D); this was also observed in most of the other species. Fiber walls of the early wood showed lignin loss, revealed by their loss of fluorescence and red color; and vessels and parenchymatous rays showed structure loss, evidenced by the cell shape alteration (Figs. 5C, 5D, white arrows). On the contrary, the fibers of the latewood exhibited less delignification evidenced by the light blue color and unchanged thickness and structure of their walls (blue arrows). Blocks inoculated with *L. portentosus* showed a greater white fluorescence in heartwood than in sapwood, which could be related to a proportionally higher content of lignin in the first one. Also, the red color was more intense in sapwood than in heartwood rays and vessels; moreover, the red tints were more intense all along the slice in sapwood than in heartwood (Figs. 5E, 5F), which could be assumed as a higher content of cellulose.

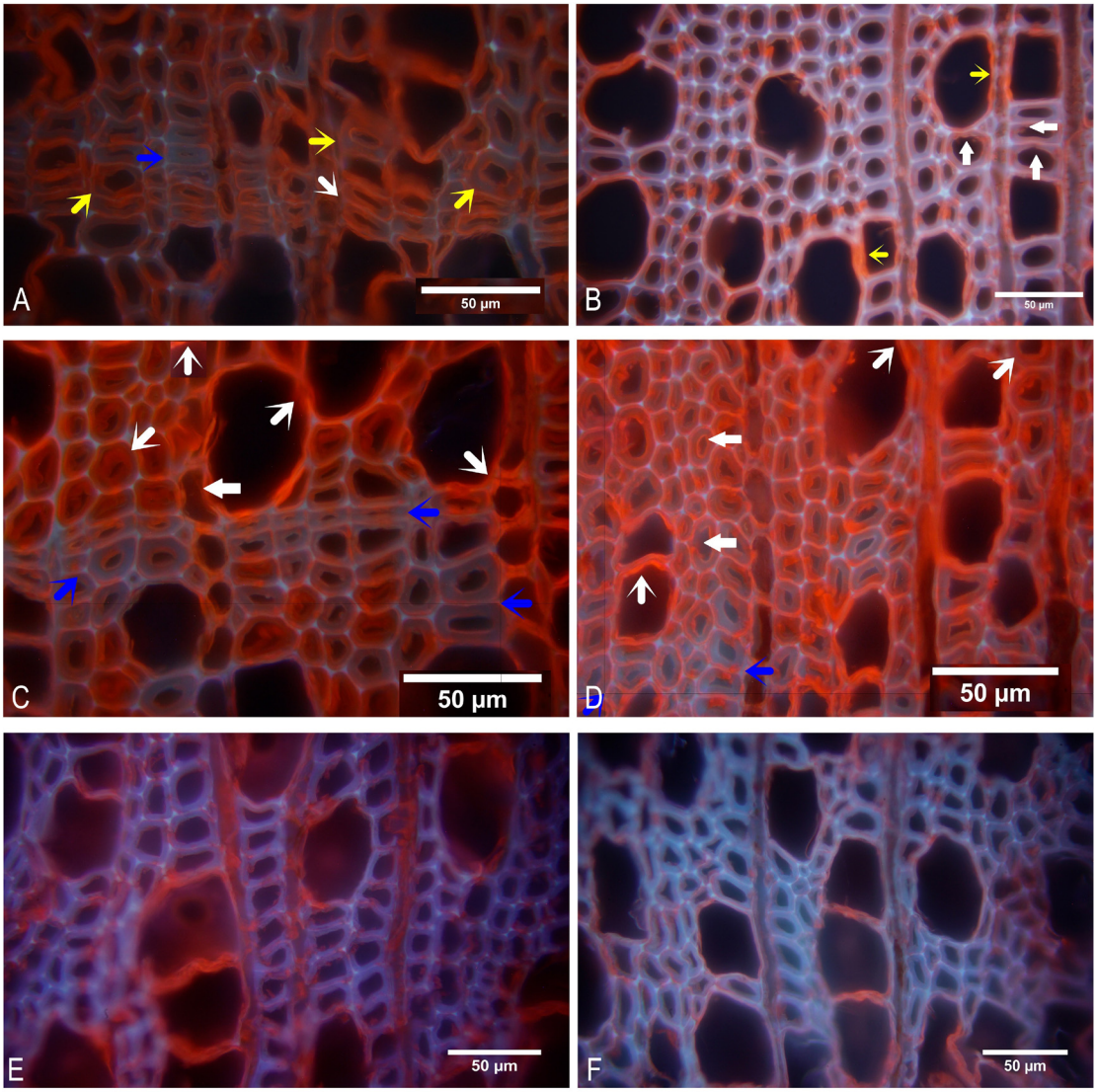

Fig. 5. Microscopic transverse sections of blocks treated with Congo red stain and photographed under UV fluorescence, and inoculated with: *Stereodiscus triviale* (A-B), *Hymenochaete australis* (C-D), and *Laetiporus portentosus* (E-F). A) Sapwood: uneven delignification with higher levels (white arrows), intermediate (yellow arrows), and lower levels (blue arrows) of degradation of the fiber walls. A red zone close to the lumen indicates that delignification starts in the inner S_3 and S_2 layers (yellow arrows). B) Heartwood: white fluorescence still remains in the middle lamella and fiber walls show blue color; however, vessels (yellow arrows) and some fibers (white arrows) show the beginning of decomposition (red color and thinning). C) Sapwood and D) Heartwood: no remarkable differences are observed between sapwood and heartwood; degradation is uneven with more (white arrows) and fewer (blue arrows) decayed areas. E) Sapwood and F) Heartwood: Sapwood's fiber walls show a darker blue color, a homogeneous reddish tint, and less white fluorescence than heartwood; it could be related to a proportionally greater content of cellulose in sapwood than in heartwood due to the greater removal of cellulose in the heartwood.

Fig. 5. Cortes transversales microscópicos de bloques tratados con colorante rojo Congo y fotografiados bajo fluorescencia UV e inoculados con: *Stereodiscus triviale* (A-B), *Hymenochaete australis* (C-D) y *Laetiporus portentosus* (E-F). A) Albura: delignificación heterogénea con paredes de fibras con alta (flechas blancas), intermedia (flechas amarillas) y baja (flechas azules) degradación. Una zona roja cerca del lumen indica que la delignificación comienza en las capas internas S_3 y S_2 (flechas amarillas). B) Duramen: aún persiste fluorescencia blanca en la laminilla media y las paredes de las fibras muestran color azul; sin embargo, los vasos (flechas amarillas) y algunas fibras (flechas blancas) muestran el inicio de la descomposición (coloración roja y adelgazamiento). C) Albura y D) Duramen: no se observan diferencias destacables entre albura y duramen; la degradación es heterogénea con áreas más y menos degradadas (flechas blancas y azules, respectivamente). E) Albura y F)

➤ Duramen: Las paredes de las fibras de la albura muestran un color azul más oscuro, un tinte rojizo homogéneo y menos fluorescencia blanca que las del duramen; lo que podría estar relacionado con un contenido proporcionalmente mayor de celulosa en la albura que en el duramen debido a la mayor remoción de celulosa en el duramen.

The phloroglucinol staining showed to be less informative than that using congo red. The staining of the fibers using phloroglucinol was heterogeneous along the annual growth rings and within each ring, both in inoculated and in control blocks (Fig. 6; Fig. 7). Within each ring, a more intense staining was observed in the latewood than in the early wood, which is consistent with the greater lignification of the latewood (Figs. 6A, 6B, white arrows). Inoculated blocks showed less staining in phloroglucinol than control blocks (Fig. 6; Fig. 7) which is consistent with a delignification process, but this was also observed, to a lesser extent, in the case of brown-rot fungi. Although this staining variation was observed along the slice, the color intensity was congruent with the values of mass loss: White-rot species with the best performance in sapwood showed less stain intensity in sapwood than in heartwood (Figs. 6 A-F; Figs. 7A, 7B). On the other hand, the diminished staining in phloroglucinol observed in blocks inoculated with brown-rot species, as in the case of *L. portentosus* (Figs. 7E, 7F), could be related to a chemical alteration of the lignin structure induced by the fungus. Previous studies that examined brown-rot in wood blocks reported that most hyphae grew on the surface of S_3 layer. As relatively large ectoenzymes of brown-rot fungi must first diffuse very deeply into the cell wall through the S_3 layer, to degrade the cellulose-rich S_2 layer, probably other compounds such as hydrogen peroxide and iron ions breach inside the cell wall and overcome the lignocellulose matrix by oxidative depolymerization thus facilitating the ectoenzymes diffusion (Schwarze, 2007).

Congo red staining, especially when observed in UV, was useful to detect lignin loss because delignified zones were revealed. In these slices, lignin autofluorescence coexisted with the red color of the stain; the balance between these two components allowed the observation of different decay degrees. Moreover, identification of more degraded zones could be done. Besides, although phloroglucinol stain allowed lignin detection, its quantification was more difficult.

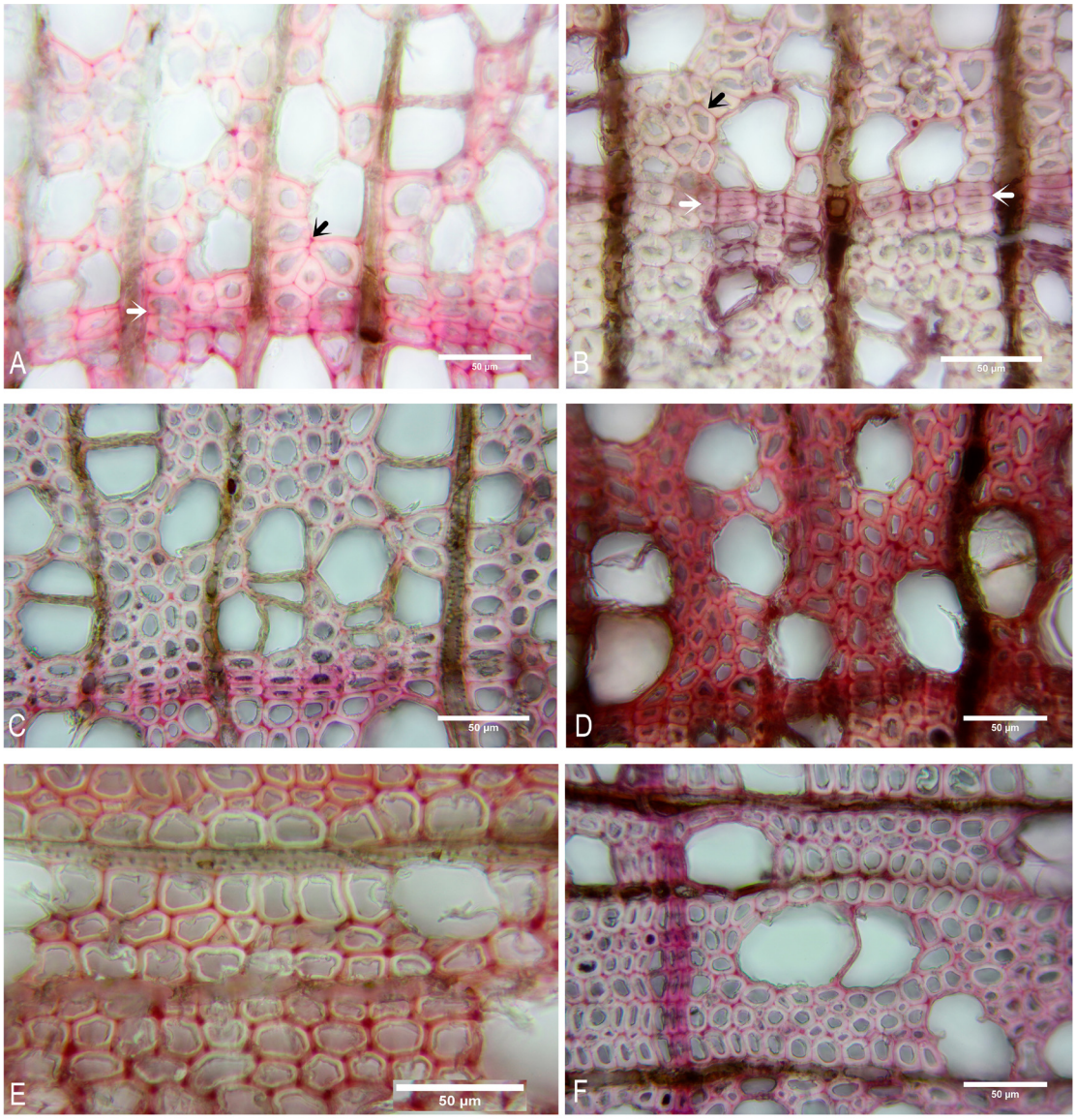

Fig. 6. Microscopic transverse sections of blocks treated with phloroglucinol stain and inoculated with: *Aurantiporus albidus* (A-B), *Rhizochaete brunnea* (C-D) and *Phanerochaete velutina* (E-F). All blocks showed a lighter pink staining than control blocks (Fig. 3C, D), indicating delignification. The greatest lignin contents were in the middle lamella (A, B, black arrows) and latewood (A, B, white arrows). A greater delignification was observed in sapwood (C) than in heartwood (D) blocks inoculated with *Rh. brunnea*, but not in blocks inoculated with *A. albidus* and *Ph. velutina.*

Fig. 6. Cortes transversales microscópicos de bloques tratados con tinción de floroglucinol e inoculados con: *Aurantiporus albidus* (A-B), *Rhizochaete brunnea* (C-D) y *Phanerochaete velutina* (E-F). Todos los bloques mostraron una tinción rosa más clara que los bloques de control (Fig. 3C, D), lo que indica delignificación. Los mayores contenidos de lignina se encontraron en la laminilla media (A, B, flechas negras) y en el leño tardío (A, B, flechas blancas). Se observó una mayor delignificación en los bloques de albura (C) que en los de duramen (D) inoculados con *Rh. brunnea*, pero no en bloques inoculados con *A. albidus* y *Ph. Velutina.*

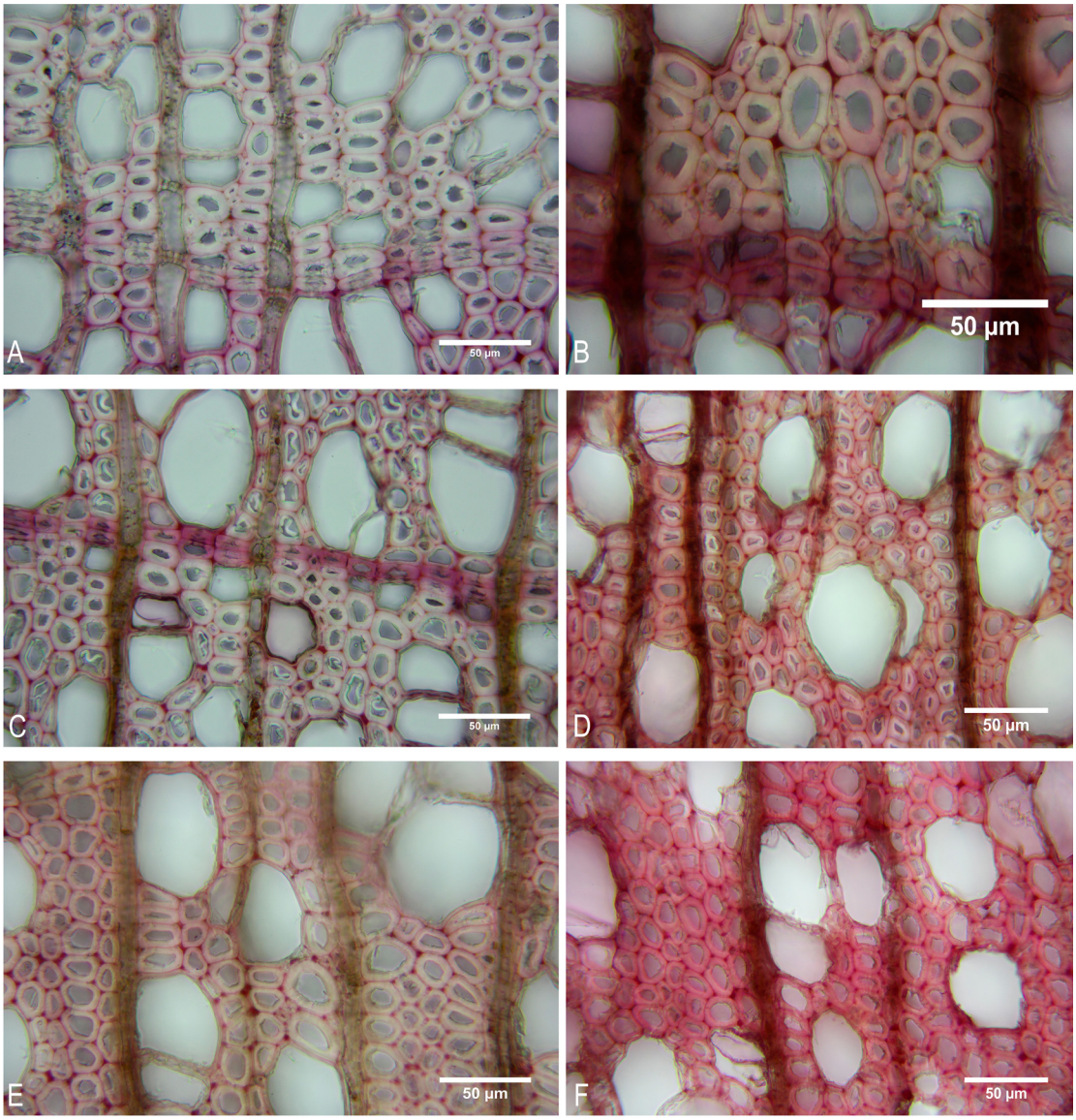

Fig. 7. Microscopic transverse sections of blocks treated with phloroglucinol stain and inoculated with: *Stereodiscus triviale* (A-B), *Hymenochaete australis* (C-D) and *Laetiporus portentosus* (E-F). All blocks showed a lighter pink staining than control blocks (Figs. 3C, D), and a lighter staining in sapwood than in heartwood. The greatest lignin contents were in the middle lamella.

Fig. 7. Cortes transversales microscópicos de bloques tratados con tinción de floroglucinol e inoculados con: *Stereodiscus triviale* (A-B), *Hymenochaete australis* (C-D) y *Laetiporus portentosus* (E-F). Todos los bloques mostraron una coloración rosa más clara que los bloques de control (Figs. 3C, D) y una coloración más clara en la albura que en el duramen. Los mayores contenidos de lignina se encontraron en la laminilla media.

CONCLUSIONS

Fungal ability to decay wood is generally studied without distinguishing between sapwood and heartwood. Our work, one of the few that analyzes the ability of different fungi to decay both regions of the wood, showed that over 60% of the species studied had significantly different performance in sapwood and heartwood. As far as we know, the ability to decay sapwood and heartwood of *Nothofagus pumilio* by

the species studied in this work is compared for the first time. Most of the white-rot fungi tested showed a preference for sapwood and all brown-rot fungi for heartwood.

Among white-rot fungi, two modes of action were observed: a generalized decay pattern, typical for *Ph. velutina*, and a localized pattern, as the one shown for *A. albidus*, *Rh. brunnea*, *S. triviale* and *H. australis*.

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