



Wood and humus decay strategies by white-rot basidiomycetes correlate with two different dye decolorization and enzyme secretion patterns on agar plates



José M. Barrasa^{a,*}, María N. Blanco^a, Fernando Esteve-Raventós^a, Alberto Altés^a, Julia Checa^a, Angel T. Martínez^b, Francisco J. Ruiz-Dueñas^b

^aDepartamento de Ciencias de la Vida, Universidad de Alcalá, E-28805 Alcalá de Henares, Spain

^bCentro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

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ABSTRACT

During several forays for ligninolytic fungi in different Spanish native forests, 35 white-rot basidiomycetes growing on dead wood (16 species from 12 genera) and leaf litter (19 species from 10 genera) were selected for their ability to decolorize two recalcitrant aromatic dyes (Reactive Blue 38 and Reactive Black 5) added to malt extract agar medium. In this study, two dye decolorization patterns were observed and correlated with two ecophysiological groups (wood and humus white-rot basidiomycetes) and three taxonomical groups (orders Polyporales, Hymenochaetales and Agaricales). Depending on the above groups, different decolorization zones were observed on the dye-containing plates, being restricted to the colony area or extending to the surrounding medium, which suggested two different decay strategies. These two strategies were related to the ability to secrete peroxidases and laccases inside (white-rot wood Polyporales, Hymenochaetales and Agaricales) and outside (white-rot humus Agaricales) of the fungal colony, as revealed by enzymatic tests performed directly on the agar plates. Similar oxidoreductases production patterns were observed when fungi were grown in the absence of dyes, although the set of enzyme released was different. All these results suggest that the decolorization patterns observed could be related with the existence of two decay strategies developed by white-rot basidiomycetes adapted to wood and leaf litter decay in the field.

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1. Introduction

Cellulose, hemicelluloses and lignin are the main components of plant cell walls produced by the photosynthetic activity of land plants. Lignin protects cellulose and hemicelluloses and is highly recalcitrant to degradation due to its aromatic nature and structural heterogeneity. This makes that lignocellulosic materials accumulate in forest soils, being their decomposition and recycling by saprotrophic fungi a key process in the global carbon cycle. A specialized group of basidiomycetes, the so called white-rot fungi, are the most efficient organisms degrading lignin,

Abbreviations: MnP, manganese peroxidase; LiP, lignin peroxidase; VP, versatile peroxidase; DyP, dye-decolorizing peroxidase; GP, generic peroxidase; RB38, Reactive Blue 38; RB5, Reactive Black 5; VA, veratryl alcohol; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

* Corresponding author.

E-mail address: josem.barrasa@uah.es (J.M. Barrasa).

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and constitute one of the most important ecophysiological groups of the mycobiota of soil in forest ecosystems. Lignocellulosic materials accumulated on forest soil are mainly represented by dead wood and leaves. According to the type of lignocellulosic material to be degraded, two types of ligninolytic basidiomycetes can be distinguished: wood and humus white-rot basidiomycetes. The former degrade wood lignin leaving a bleached substrate (Blanchette, 1995; Eriksson et al., 1990; Otjen and Blanchette, 1986) and the latter degrade lignin and polyphenols of leaves causing the so-called white-rot humus (Hintikka, 1970; Osono, 2007). Whilst white-rot wood basidiomycetes are broadly represented by members of the orders Polyporales and Hymenochaetales and some others of the order Agaricales (Worrall et al., 1997), white-rot humus basidiomycetes are mainly found among members of Agaricales (Hintikka, 1970; Osono, 2007; Steffen et al., 2002).

To degrade the complex molecule of lignin, white-rot basidiomycetes have developed an extracellular ligninolytic system,

whose composition often differs between species (Floudas et al., 2012; Ruiz-Dueñas et al., 2013). In general, it is made up of low molar-mass metabolites, oxidases and ligninolytic enzymes such as laccases and especially high redox-potential peroxidases. The latter include manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) being activated by the hydrogen peroxide produced by oxidases (Martínez et al., 2005), whose genes are characteristic of white-rot fungal genomes (Floudas et al., 2012). Recently, a dye-decolorizing peroxidase (DyP) from the white-rot basidiomycete *Auricularia auricula-judae* has been demonstrated to oxidize a non-phenolic lignin model dimer (Liers et al., 2010). This fact, together with the higher number of DyP genes in genomes of wood white-rot fungi compared with brown-rot fungi (Floudas et al., 2012; Ruiz-Dueñas et al., 2013) suggest that this type of peroxidases may be also involved in lignin biodegradation.

Ligninolytic peroxidases and laccases have a broad substrate specificity acting directly or through mediators (Cañas and Camarero, 2010; Ruiz-Dueñas et al., 2009). The same enzymes can also degrade recalcitrant aromatic compounds (Bumpus et al., 1985; Pointing, 2001; Reddy, 1995) including synthetic aromatic dyes used in textile industries (Barrasa et al., 2009; Jarosz-Wilkolazka et al., 2002; Knapp et al., 1995; McMullan et al., 2001; Toh et al., 2003), some of which have been used to detect ligninolytic activity in culture (Gold et al., 1988). The above studies were mainly focused on wood decaying fungi, and only a few members of the group of leaf litter decomposing fungi were tested for dye decolorization (Jarosz-Wilkolazka et al., 2002). In a similar way, a fungal screening for oxidative enzymes involved in lignin degradation was carried out by Peláez et al. (1995), in which a total of 68 species belonging to different families of basidiomycetes was studied, however, a scarce number of leaf litter decomposing species was included.

In the present work, a screening of white-rot basidiomycetes growing on dead wood and leaf litter is reported by analyzing their ability to decolorize two recalcitrant aromatic dyes on agar plates, the phthalocyanine dye Reactive Blue 38 (RB38) and the azo dye Reactive Black 5 (RB5), and two decolorization patterns are described. Although fungal enzymes are generally studied in liquid media, different oxidoreductases have also been identified in agar plate cultures, being often related to the wood decay patterns caused by basidiomycetes (Käärik, 1965; Liers et al., 2011; Moukha et al., 1993; Stalpers, 1978; Westermarck and Eriksson, 1974). Here, assays to detect high and low redox potential oxidoreductase activities on agar plates, with and without dyes, were carried out on representative white-rot wood and humus basidiomycetes. The decolorization patterns and enzymes produced were finally correlated with two possible strategies followed by white-rot fungi to colonize wood and humus. This wide screening will contribute to our knowledge of the diversity of fungi degrading lignocellulose in Spanish native forest, and their involvement in wood and leaf litter decomposition.

2. Materials and methods

2.1. Fungal sampling and culture

A total of 166 fungal strains from 123 species of basidiomycetes fruiting on dead wood and leaf litter were isolated after eight fungal forays in different Spanish native forests. The collected samples consisted of fresh fruit bodies, which were conserved at 4 °C before grown as pure cultures. Fungal isolations were made from mycelium (5 × 5 mm pieces of context) aseptically removed from fruit bodies (Peláez et al., 1992) that was inoculated in Petri dishes with

malt extract (2% wt/vol) agar (MEA; Pronadisa) containing ampicillin (1.5 mg/l; Sigma).

Microscope examination of hyphae were performed for the presence of clamp connections confirming their basidiomycetous nature. Size and color of the colony, shape and size of hyphae and type of septa were also checked (Stalpers, 1978). Fungal taxonomy was considered according to phylogenetic studies (Binder et al., 2005, 2013; Hibbett et al., 2007; Matheny et al., 2006). The type of rot was determined by the color and characteristics of wood (Rayner and Boddy, 1988) and leaf litter (Osono, 2007) close to the fungal fruit body. Fungal dry material is deposited in AH (Herbario de la Universidad de Alcalá) and pure cultures are conserved in the fungal culture collection of Departamento de Ciencias de la Vida (Universidad de Alcalá).

2.2. Dye decolorization assays in agar plates

Decolorization assays were carried out on Petri dishes (9 cm diameter) with 20 ml of MEA containing RB38 ($C_{32}N_8NiH_{12}(SO_3H)_n(SO_2-NH-C_6H_4-reactive\ group)_{4-n})$ and RB5 ($C_{26}H_{21}N_5O_{19}S_6Na_4$) (both from Sigma) at two concentrations (75 and 150 mg/l) (Fig. 1). Plugs of 0.5 cm diameter from MEA cultures were inoculated on plates with the synthetic dyes, which were incubated at 25 °C and examined each 2 days for decolorization. The radial mycelial growth and decolorized area were measured, and only those species producing a decolorization circle of at least 2 cm diameter, within 15 days of incubation, were selected.

2.3. Enzymatic assays in plates

Enzymatic activities were tested directly on fungal colonies grown (at 25 °C) on: (i) MEA plates; and (ii) RB5 and RB38 containing MEA, where the fungi had previously decolorized these dyes, as described in Section 2.2.

With this purpose, 10 µl of 0.1 M *o*-hydroxyphenol (catechol, Merck), *o*-methoxyphenol (guaiacol, Merck), 2,6-dimethoxyphenol (syringol, Merck) or 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Roche Applied Sciences) in 96% (vol/vol) ethanol were dropped on MEA plate cultures in front of, behind or on the edge of the fungal colony. These substrates are oxidized by laccase (EC 1.10.3.2) (Baldrian, 2006), and by generic peroxidase (GP; EC 1.11.1.7), short MnP, VP and DyP (EC 1.11.1.13, 1.11.1.16 and 1.11.1.19, respectively) in the presence of hydrogen peroxide (Hofrichter et al., 2010; Morales et al., 2012).

Ten µl of 0.1 M RB5 or 25 mM RB38 in 96% (vol/vol) ethanol, which are oxidized by VP and DyP in the presence of hydrogen peroxide (Heinfling et al., 1998b; Liers et al., 2010), were assayed both on MEA plates, as previously described for phenols and ABTS, and on decolorized MEA plates. ABTS, RB5 and RB38 were not completely soluble under the above conditions and they were added as a suspension on the plate. Enzymatic oxidation of dyes mediated by 3,4-dimethoxybenzyl (veratryl) alcohol (VA, Sigma) radical resulting from VA oxidation by LiP (EC 1.11.1.14) and VP, was also examined (Tinoco et al., 2007). With this purpose, 10 µl of 20 mM VA (in H₂O) were added together with 10 µl of 0.1 M RB5 or 25 mM RB38 prepared in 96% (vol/vol) ethanol.

Five µl of a concentrated catalase (Sigma) solution was also supplied prior to the addition of the above substrates to remove any peroxide traces and avoid peroxidase activity. Alternatively, 1 µl of 10 mM hydrogen peroxide (Merck) was simultaneously added together with dyes, ABTS or phenols to confirm the presence of peroxidases. Substrate oxidation was easily followed by changes in color.

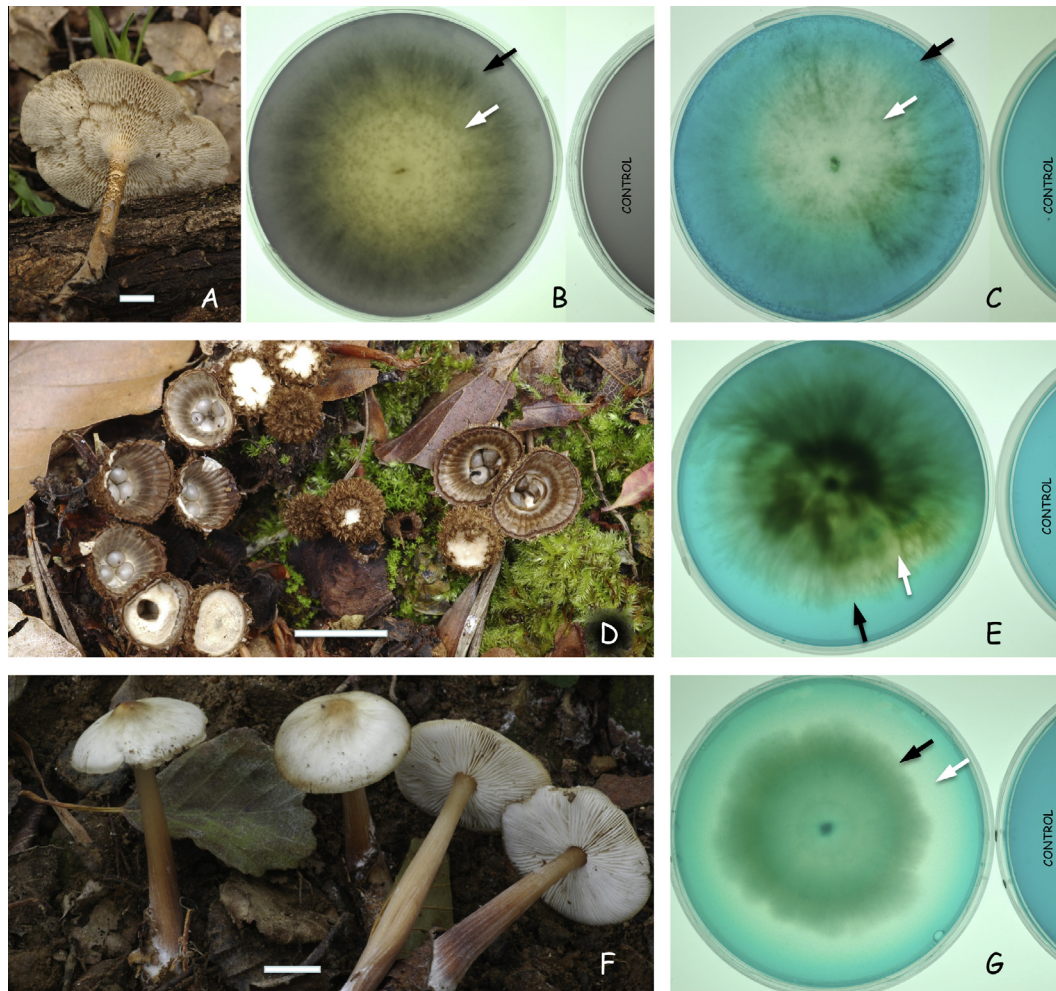


Fig. 1. Agar-plate dye (150 mg/ml) decolorization patterns. (A–C) *Polyporus arcularius* (AH 44709): (A) Basidiocarp. (B, C) Dye decolorization inside the fungal colony (white-rot wood decolorization pattern): (B) Culture in MEA + Reactive Black 5. (C) Culture in MEA + Reactive Blue 38. (D, E) *Cyathus striatus* (AH 40144): (D) Basidiocarps. (E) Dye decolorization inside the fungal colony (white-rot wood decolorization pattern), culture in MEA + Reactive Blue 38. (F, G) *Rhodocollybia butyracea* (AH 40177): (F) Basidiocarps. (G) Dye decolorization outside the fungal colony (white-rot humus decolorization pattern), culture in MEA + Reactive Blue 38. Black arrows = edge of fungal colonies. White arrows = decolorization areas. Bars = 10 mm.

3. Results

3.1. Fungal species and their phylogenetic position

Pure cultures of 123 species of white-rot basidiomycetes isolated from dead wood and soil litter were investigated for their ability to decolorize two recalcitrant aromatic dyes (RB38 and RB5) on MEA plates. Among them, a total of 35 species were selected because of the ability to produce a decolorization circle of at least 2 cm diameter within 15 days of incubation (Table 1). Polyporales and Hymenochaetales were more efficient since they decolorized both dyes at the two concentrations assayed (75 and 150 mg/l) within 8 days, while Agaricales decolorized only RB38 at 15 days of incubation. Brown-rot species did not decolorize the dyes and are not considered in this work. The selected species belong to three taxonomic orders – namely Polyporales, Hymenochaetales and Agaricales – and different phylogenetic clades.

3.2. Fungal lifestyle and decay patterns

The 35 selected species could be divided in two ecophysiological groups. The first group includes 16 species characterized by

their ability to colonize and degrade dead wood in forest, mainly represented by trunks and stumps, causing a white-rot decay pattern as a consequence of lignin removal. The second group includes 19 species of leaf litter decomposing fungi, characterized by their ability to colonize and degrade dead leaves contributing to soil humus formation. The white-rot humus decay pattern results in bleaching of brown dead leaves due to polyphenol and lignin degradation. The majority of the wood decomposing species studied belongs to the order Polyporales (11) but a few of them belong to the orders Agaricales (4) and Hymenochaetales (1). Otherwise, all the humus basidiomycetes investigated (19 species) belong to the order Agaricales, most of them included in Marasmiaceae, Mycenaceae and Strophariaceae families in agreement with previous studies (Hintikka, 1988; Osono, 2007; Osono and Takeda, 2006; Tyler, 1985). The species of Agaricales degrading wood are mainly included in the Physalacriaceae family, although some members of Agaricaceae (i.e. *Cyathus striatus*) and Strophariaceae (i.e. *Gymnopilus junonius*, synonym: *G. spectabilis* sensu auct.) have also developed a wood decomposing lifestyle, in agreement with Jahn (1979) (Table 1).

Ten of the eleven white-rot Polyporales species were found on angiosperm wood. The only exception was *Abortiporus biennis* that, as the only species of Hymenochaetales studied (*Sidera lenis*), was

Table 1

Selected species decolorizing agar-plates dyes (decolorization ring = 2 cm diameter) during 15 days of incubation.

N°AH	Species	Family	Order	Phylogenetic position	Substrate, Location	RB38	RB5	Days required for decolorization		Decolorization pattern ^a
								RB38	RB5	
44723	<i>Abortiporus biennis</i> (Bull.) Singer	Meruliaceae	Polyporales	Residual polyporoid clade	Wood (<i>Pinus radiata</i>), Pared Vieja (La Palma)	+	+	8	8	WRW
44705	<i>Corioloopsis gallica</i> (Fr.) Ryvar den	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Acer platanoides</i>), Pedrezuela (Madrid)	+	+	4	4	WRW
44712	<i>Corioloopsis gallica</i> (Fr.) Ryvar den	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	+	6	6	WRW
44706	<i>Corioloopsis trogii</i> (Berk.) Domański	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Populus alba</i>), Alcalá de Henares (Madrid)	+	+	5	5	WRW
44707	<i>Ganoderma resinaceum</i> Boud.	Ganodermataceae	Polyporales	Core polyporoid clade	Wood (<i>Ligustrum lucidum</i>), Alcalá de Henares (Madrid)	+	+	5	5	WRW
44713	<i>Ganoderma australe</i> (Fr.) Pat.	Ganodermataceae	Polyporales	Core polyporoid clade	Wood (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	+	6	6	WRW
44708	<i>Phlebia radiata</i> Fr.	Meruliaceae	Polyporales	Phlebiod clade	Wood (<i>Ilex aquifolium</i>), Robregordo (Madrid)	+	+	3	3	WRW
44716	<i>Phlebia rufa</i> (Pers.) M.P. Christ.	Meruliaceae	Polyporales	Phlebiod clade	Wood (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	+	8	8	WRW
44709	<i>Polyporus arcularius</i> (Batsch) Fr.	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Prunus lusitánica</i>), Las Villuercas (Cáceres)	+	+	6	7	WRW
44704	<i>Trametes pubescens</i> (Schumach.) Pilát	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Laurus azorica</i>), Pared Vieja (La Palma)	+	+	8	8	WRW
44710	<i>Trametes versicolor</i> (L.) Lloyd	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Prunus lusitánica</i>), Las Villuercas (Cáceres)	+	+	5	5	WRW
44714	<i>Trametes versicolor</i> (L.) Lloyd	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	+	8	6	WRW
44715	<i>Trametes versicolor</i> (L.) Lloyd	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	+	8	6	WRW
44711	<i>Trametes versicolor</i> (L.) Lloyd	Polyporaceae	Polyporales	Core polyporoid clade	Wood (<i>Ocotea foetens</i>), Pared Vieja (La Palma)	+	+	8	8	WRW
44717	<i>Steccherinum ochraceum</i> (Pers.) Gray	Steccherinaceae	Polyporales	Residual polyporoid clade	Wood (<i>Alnus glutinosa</i>), Las Batuecas (Salamanca)	+	–	10	–	WRW
44703	<i>Sidera lenis</i> (P. Karst.) Miettinen	Repetobasidiaceae	Hymenochaetales	Hymenochaetoid clade	Wood (<i>Pinus radiata</i>), Pared Vieja (La Palma)	+	+	6	8	WRW
40210	<i>Agrocybe pediades</i> (Fr.) Fayod	Strophariaceae	Agaricales	Agaricoid clade	Humus (Grass), Cantalojas (Guadalajara)	+	–	14	–	WRH
40215	<i>Agrocybe praecox</i> (Pers.) Fayod	Strophariaceae	Agaricales	Agaricoid clade	Humus (<i>Betula pendula</i>), Cantalojas (Guadalajara)	+	–	12	–	WRH
40153	<i>Crucibulum laeve</i> (Huds.) Kambly	Nidulariaceae	Agaricales	Agaricoid clade	Humus (<i>Fagus sylvatica</i>), Selva de Oza (Huesca)	+	–	10	–	WRH
44718	<i>Cyathus olla</i> (Batsch) Pers.	Nidulariaceae	Agaricales	Agaricoid clade	Humus (<i>Pinus halepensis</i>), Alcalá de Henares (Madrid)	+	–	5	–	WRH
40144	<i>Cyathus striatus</i> (Huds.) Willd.	Nidulariaceae	Agaricales	Agaricoid clade	Wood (<i>Fagus sylvatica</i>), Belagua (Navarra)	+	–	15	–	WRW
44721	<i>Gymnopilus junonius</i> (Fr.) P.D. Orton	Strophariaceae	Agaricales	Agaricoid clade	Wood (<i>Pinus radiata</i>), Pared Vieja (La Palma)	+	–	15	–	WRW
40209	<i>Gymnopus aquosus</i> (Bull.) Antonín & Noordel.	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Betula pendula</i>), Cantalojas (Guadalajara)	+	–	15	–	WRH
40138	<i>Gymnopus confluens</i> (Pers.) Antonín, Halling & Noordel.	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Fagus sylvatica</i>), Selva de Irati (Navarra)	+	–	15	–	WRH
40214	<i>Gymnopus dryophilus</i> (Bull.) Murrill	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Fagus sylvatica</i>), Cantalojas (Guadalajara)	+	–	10	–	WRH
40212	<i>Gymnopus ocior</i> (Pers.) Antonín & Noordel.	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Betula pendula</i>), Cantalojas (Guadalajara)	+	–	6	–	WRH
40270	<i>Lepista nuda</i> (Bull.) Cooke	Tricholomataceae	Agaricales	Tricholomatoid clade	Humus (<i>Pinus halepensis</i>), Alcalá de Henares (Madrid)	+	–	8	–	WRH
40158	<i>Lycoperdon</i> sp.	Agaricaceae	Agaricales	Agaricoid clade	Humus (<i>Fagus sylvatica</i>), Bosque de Gamueta (Huesca)	+	–	6	–	WRH
44722	<i>Lycoperdon</i> sp.	Agaricaceae	Agaricales	Agaricoid clade	Humus (<i>Ocotea foetens</i>), Cubo de la Galga (La Palma)	+	–	5	–	WRH
44720	<i>Macrocyttidia cucumis</i> (Pers.) Joss.	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Laurus azorica</i>), Pared Vieja (La Palma)	+	–	8	–	WRH
40150	<i>Mycena arcangeliana</i> Bres.	Mycenaceae	Agaricales	Tricholomatoid clade	Humus (<i>Fagus sylvatica</i>), Bosque de Gamueta (Huesca)	+	–	8	–	WRH
40321	<i>Mycena leptoccephala</i> (Pers.) Gillet	Mycenaceae	Agaricales	Tricholomatoid clade	Humus (<i>Pinus halepensis</i>), Alcalá de Henares (Madrid)	+	–	10	–	WRH
40131	<i>Mycena polygramma</i> f. <i>pumila</i> J.E. Lange	Mycenaceae	Agaricales	Tricholomatoid clade	Humus (<i>Fagus sylvatica</i>), Selva de Irati (Navarra)	+	–	8	–	WRH
40185	<i>Mycena rosea</i> Gramberg	Mycenaceae	Agaricales	Tricholomatoid clade	Humus (<i>Castanea sativa</i>), Las Batuecas (Salamanca)	+	–	9	–	WRH
44719	<i>Mycena</i> sp.	Mycenaceae	Agaricales	Tricholomatoid clade	Humus (<i>Ocotea foetens</i>), Los Tiles (La Palma)	+	–	15	–	WRH

(continued on next page)

Table 1 (continued)

N°AH	Species	Family	Order	Phylogenetic position	Substrate, Location	RB38	RB5	Days required for decolorization		Decolorization pattern ^a
								RB38	RB5	
36488	<i>Oudemansiella mucida</i> (Schr.) Höhn	Physalacriaceae	Agaricales	Marasmioid clade	Wood (<i>Fagus sylvatica</i>), Cantalojas (Guadalajara)	+	–	12	–	WRW
19073	<i>Pholiota conissans</i> (Fr.) M.M. Moser	Strophariaceae	Agaricales	Agaricoid clade	Humus (Grass), Cabañeros (Ciudad Real)	+	–	5	–	WRH
40177	<i>Rhodocollybia butyracea</i> (Bull.) Lennox	Marasmiaceae	Agaricales	Marasmioid clade	Humus (<i>Castanea sativa</i>), Las Batuecas (Salamanca)	+	–	8	–	WRH
40137	<i>Xerula radicata</i> (Relhan) Dörfelt	Physalacriaceae	Agaricales	Marasmioid clade	Wood (<i>Fagus sylvatica</i>), Selva de Irati (Navarra)	+	–	14	–	WRW

^a WRW = white-rot wood decolorization pattern. WRH = white-rot humus decolorization pattern.

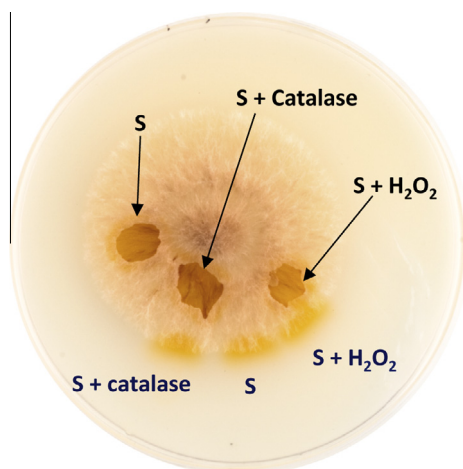


Fig. 2. Syringol (S) oxidation catalyzed by *Cyathus striatus* (AH 40144) (white-rot wood basidiomycete) grown on MEA plate. Syringol was added alone (S) and in the presence of hydrogen peroxide (S + H₂O₂) or catalase (S + catalase). The three reactions were initiated at the same time, both within and on the edge of the fungal colony.

found on coniferous wood. Among the wood-decomposing Agaricales, three species (*Oudemansiella mucida*, *Xerula radicata* and *C. striatus*) were found on angiosperm wood and one species (*G. junonius*) was found on conifer wood.

3.3. Dye plate decolorization patterns

Two different decolorization patterns were observed when the selected fungal species were grown on MEA plates containing the two dyes assayed (Table 1 and Fig. 1). On one hand, the white-rot wood fungi were characterized by the formation of a decolorized circle inside the colony. This pattern was observed in wood Polyporales, Hymenochaetales and Agaricales. On the other hand, white-rot humus fungi exhibited a decolorization pattern characterized by the formation of a decolorized circle outside the colony. This pattern was observed in humus Agaricales.

With the only exception of *Steccherinum ochraceum*, which was unable to degrade RB5, all species of Polyporales, and *S. lenis* in Hymenochaetales, were more efficient decolorizers than Agaricales, since they degraded both dyes at the highest concentration in less than 8 days. The four species of wood Agaricales (*C. striatus*, *G. junonius*, *O. mucida* and *X. radicata*) were less efficient than the wood Polyporales and Hymenochaetales since they required more than 11 days to produce a 2 cm circle in RB38 plates. Among Polyporales, *Phlebia radiata* (Meruliaceae) was the strongest dye decolorizer since in all the cases it caused 2 cm decolorization at 3 days of incubation. Similar results were obtained with *Coriopsis*

gallica (strain AH 44705), *Coriopsis trogii*, *Ganoderma resinaceum* and *Trametes versicolor* (strain AH 44710) at 4–5 days of incubation.

The two ecophysiological groups of Agaricales, namely white-rot wood and humus species, decolorized only RB38. Most of them needed 8 or more days of incubation to attain 2 cm decolorization in the plates with the highest dye concentration. Nevertheless, *Cyathus olla* (Nidulariaceae), *Lycoperdon* sp. (AH 44722; Agaricales) and *Pholiota conissans* (Strophariaceae) were able to produce a decolorization circle of 2 cm diameter in plates with RB38 in 5 days of incubation.

3.4. In situ activity tests on different substrates added to MEA cultures

Enzymatic assays were performed directly on MEA plates where ten species representative of the main phylogenetic clades (see Table 1) of white-rot wood Polyporales (*Polyporus arcularius*, *Phlebia rufa*, *Trametes pubescens* and *A. biennis*), white-rot wood Agaricales (*X. radicata*, *C. striatus* and *G. junonius*) and white-rot humus Agaricales (*Mycena polygramma* f. *pumila*, *Rhodocollybia butyracea* and *Agrocybe pediades*) under study had been previously grown. The four wood Polyporales and the three wood Agaricales were able to oxidize all the low redox potential substrates (i.e. catechol, guaiacol, syringol and ABTS) added within and on the inner edge of the fungal colony (Fig. 2 shows syringol oxidation by *C. striatus*) but not the two recalcitrant dyes (RB5 and RB38) even after addition of exogenous hydrogen peroxide and VA (the latter as a potential mediator). Peroxidase involvement was definitively ruled out, since the addition of catalase did not produce any significant effect on the oxidation of phenols and ABTS, and the reaction could be associated to laccase type activity.

Similarly, the three humus Agaricales were able to oxidize the three phenols and ABTS and unable to decolorize RB5 and RB38. However, unlike that observed with the wood basidiomycetes, the oxidative activity was mainly detected on the outer edge and in front of the limits of the fungal colony (Fig. 3 shows syringol oxidation by *A. pediades*). Oxidation of ABTS and syringol was slowed down, but not completely removed, in the presence of catalase, and conversely it was accelerated when peroxide was added. According to these results, laccases would be responsible for the low activity levels observed in the presence of catalase. We could also confirm both that peroxide is generated by these fungi, and that peroxidases are produced. In addition, we could identify this activity as low redox potential generic peroxidase considering its inability to oxidize RB5 and RB38 (even in the presence of VA).

3.5. In situ activity tests on RB5 and RB38 added to dye-MEA cultures

Enzymatic assays were also performed by adding RB5 and RB38 on dye plates where the dyes had been previously decolorized by the fungi.

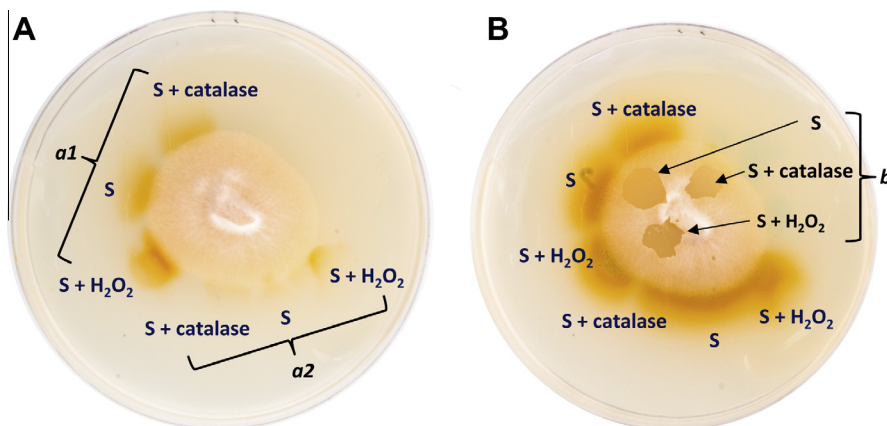


Fig. 3. Syringol (S) oxidation catalyzed by *Agrocybe pediades* (AH 40210) (white-rot humus basidiomycete) grown on MEA plate. Syringol was added alone (S) and in the presence of hydrogen peroxide (S + H₂O₂) or catalase (S + catalase). The above three reactions were initiated at the same time and were tested in a single plate, first in front of (A) and then within (B) the limits of the fungal colony, in the following order: (i) *a1* and then *a2* (in front of the edge of the colony); and (ii) *b* (within the colony). *a2* reactions were initiated with a 10 min delay with respect to the *a1* onset and both were developed 5 min later. Syringol oxidation in *a1* and *a2* was faster in the presence of hydrogen peroxide and slower in the presence of catalase. The *a1* and *a2* reactions are saturated in (B).

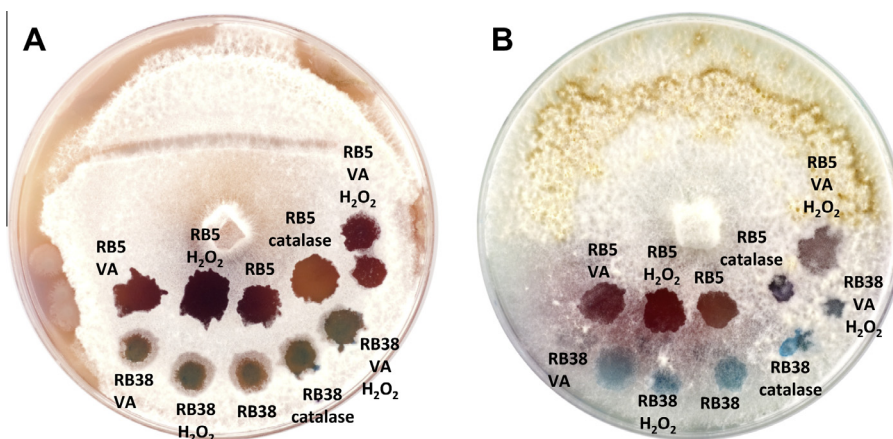


Fig. 4. Reactive Black 5 (RB5) and Reactive Blue 38 (RB38) oxidation assays in MEA plates where *Trametes pubescens* (AH 44704) (white-rot wood basidiomycete) has previously decolorized these two dyes (RB5 in (A) and RB38 in (B)). Both dyes were added alone or together with catalase, hydrogen peroxide, veratryl alcohol (VA) or VA plus hydrogen peroxide, as described in Section 2. RB38 was not oxidized under any of the conditions tested and RB5 oxidation was evidenced by formation of a reddish-brown product after 16 and 48 h incubation in plates (A) and (B) respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

C. striatus, tested as representative of white-rot wood Agaricales, was unable to decolorize any of the dyes when added on previously decolorized RB38 plates (even after several days of subsequent incubation). The addition of the dyes was supplemented with hydrogen peroxide and VA without positive results. The above findings confirm the absence of any enzymatic activity able to oxidize these dyes, such as ligninolytic peroxidases and DyPs.

On the other hand, *T. pubescens*, as representative of wood Polyporales, was able to decolorize RB5 but not RB38 when added on the previously decolorized dye plates. In the RB5 plates, high decolorization activity was observed 16 h after RB5 addition within the colony (Fig. 4A). This ability was enhanced by hydrogen peroxide and dramatically impaired, although not completely removed, by catalase (VA did not produce any effect). These results confirm the involvement of both laccase (maybe acting through natural mediators) and peroxidase activities in the oxidation of this dye, as well as the fungal production of the hydrogen peroxide necessary for peroxidase activity. In the RB38 plates, no decolorization of RB5 was observed up to 2 days after its addition (Fig. 4B). A

6 day period was necessary when catalase was simultaneously added. Again, these results suggest the production of both hydrogen peroxide and peroxidase activity. Moreover, the long time (days) necessary to decolorize RB5 suggests *de novo* synthesis of the enzymes involved, unlike that observed on the RB5 plates where substrate oxidation only lasted minutes (or a few hours). Delayed decolorization by laccase and peroxidase activities was also observed with *A. biennis*, a second representative of wood Polyporales, although at longer incubation times (data not shown).

Finally, *R. butyracea* was tested as a representative for the white-rot humus Agaricales. This species was unable to decolorize any of the recalcitrant dyes when added alone or together with peroxide or catalase in the inner part of the colony grown on dye-containing MEA (Fig. 5A). However, both RB5 and RB38 were completely decolorized at the edge and in front of the limits of the colony when incubation was extended to 20 h, as observed under all the experimental conditions assayed except in the presence of catalase (Fig. 5B). These results suggest *de novo* enzyme synthesis, as mentioned above, and confirm the importance of hydrogen peroxide in the decolorization process by this fungus.

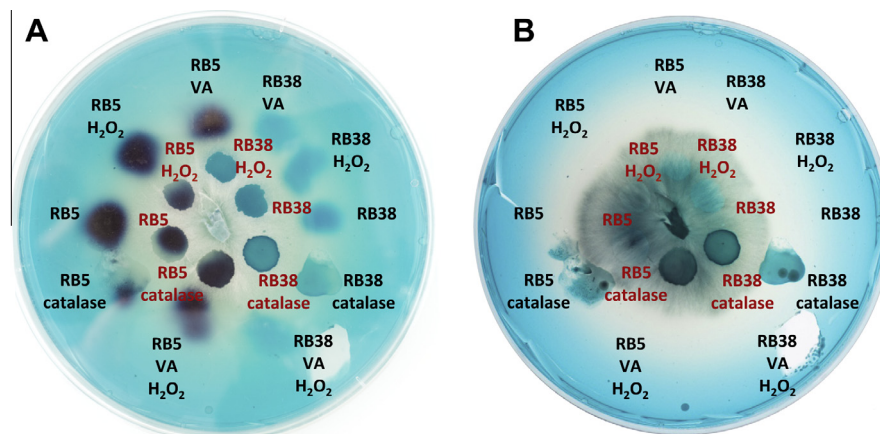


Fig. 5. Reactive Black 5 (RB5) and Reactive Blue 38 (RB38) oxidation assays in MEA plate where RB38 has been previously decolorized by *Rhodocollybia butyracea* (AH 40177) (white-rot humus basidiomycete). (A) Both dyes (still not oxidized) immediately after added alone or together with catalase, hydrogen peroxide, veratryl alcohol (VA) or VA plus hydrogen peroxide on the previously decolorized area (inside, red labels, and on the edge, black labels, of the fungal colony). (B) Both dyes completely decolorized (on the edge of the colony) after 20 h incubation under all the experimental conditions tested, except when catalase was simultaneously added. Neither of the two dyes was decolorized behind the limits of the colony. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Decolorization of recalcitrant dyes by wood and humus basidiomycetes

In the present work, we describe for the first time two different decolorization patterns by white-rot basidiomycetes based on results of 35 fungal species grown on dye-containing MEA plates. White-rot basidiomycetes can be classified as either wood or leaf litter decomposers. However a few species have been reported to decompose both wood and leaf litter, such as *Hypholoma* species (Snajdr et al., 2010; Steffen, 2003). Moreover, species growing on partially decayed and buried wood (e.g. *Agrocybe aegerita*) cause a nonspecific type of rot (Dix and Webster, 1995). Therefore, further studies on the latter ecophysiological groups of fungi should be carried out for dye decolorization patterns and enzymatic activities. Interestingly, different lignin degradation and oxidoreductases secretion patterns were found, respectively, between white-rot wood, white-rot leaf litter and nonspecific wood rot basidiomycetes, growing on lignocellulosic materials (Liers et al., 2011).

A screening of 30 wood-rotting basidiomycetes of different taxonomic groups for both decolorization of RB5 and RB38 and ligninolytic activities was carried out by Barrasa et al. (2009) and only two white-rot wood Polyporales and one Dacrymycetal were reported to decolorize both dyes within 5 incubation days. Moreover, no white-rot humus basidiomycetes were included in this study and, therefore, differential decolorization patterns could not be established. On the other hand, Jarosz-Wilkolazka et al. (2002) compared dye decolorization by 115 fungal strains, but only one white-rot humus species of Agaricales and 12 wood-rotting species (8 Polyporales and 4 Agaricales) were reported to decolorize the azo dye assayed (Acid Red 183). In the present work, none of the white-rot humus species of Agaricales assayed were able to decolorize the azo dye (RB5), but 15 white-rot wood species (14 Polyporales and 1 Hymenochaetal) were able to decolorize this dye within 8 incubation days. This suggests that the azo dye is more recalcitrant to fungal degradation, and that white-rot wood Polyporales and Hymenochaetales shows higher ability to decolorize it. However, after grown on RB38 plates, some white-rot humus Agaricales (i.e. *R. butyracea*) seem to be prepared to decolorize more recalcitrant dyes, such as RB5, by a mechanism that should be more in depth investigated.

4.2. Ecophysiological groups and dye-decolorizing enzymes in white-rot basidiomycetes

This is also the first time that a correlation between ecophysiological groups of lignin degrading basidiomycetes and dye decolorization patterns is observed. Dye decolorization is a simple assay for the identification of fungal ability to transform lignin. Both lignin and the recalcitrant dyes assayed are aromatic compounds that can be oxidized by ligninolytic peroxidases in the presence of hydrogen peroxide (Heinfling et al., 1998a; Ollikka et al., 1993; Tinoco et al., 2007). This reaction has been described to be catalyzed directly or in the presence of low molecular mass compounds acting as mediators (Ruiz-Dueñas and Martínez, 2009). Among them, VA was the first aromatic compound described as a natural mediator in cultures of the model white-rot fungus *Phanerochaete chrysosporium* (Harvey et al., 1986), and it is this compound which was used here to try to identify high-redox potential peroxidases that are not able to directly oxidize RB5 and RB38. DyPs were the second superfamily of high-redox potential peroxidases recently described as able to directly oxidize both recalcitrant aromatic dyes and lignin model compounds (Liers et al., 2010, 2013). Finally, laccases have been also described as able to oxidize lignin model compounds and dyes especially in the presence of mediators, including those synthesized by white-rot fungi (Cañas and Camarero, 2010). In consequence, it was expected that these extracellular enzymes should be produced in MEA plates during the decolorization of RB5 and RB38 by the white-rot basidiomycetes studied, as previously reported for several white-rot fungi growing in the presence of dyes (Casas et al., 2007; Eichlerová et al., 2005, 2006a,b; Mendonca et al., 2008; Rodríguez-Couto, 2012; Salame et al., 2012).

The enzymatic assays performed in three regions of the fungal plates – within, on the edge, and outside the limits of the fungal colony – revealed that indeed laccases and peroxidases are released in those regions where dyes are decolorized in cultures of white-rot wood Agaricales and Polyporales (within and on the inner edge of the colony) and white-rot humus Agaricales (on the outer edge and outside the limits of the colony). The *C. striatus* plates where RB5 and RB38 had been previously decolorized provided the only exception, since no enzymatic activities could be detected, most probably due to enzyme inactivation after dye decolorization. This could be due to the action of proteases, which

were reported to inactivate ligninolytic peroxidases in fungal cultures (Cabaleiro et al., 2002) and also cause laccase inactivation (Palmieri et al., 2000).

On the other hand, the enzymatic analysis on MEA plates without dyes revealed basically the same wood and humus basidiomycete secretion patterns identified on the dye plates, although the set of secreted enzymes was not competent for dye decolorization. The latter is easily understandable considering that genes encoding the wide range of peroxidases and laccases secreted by white-rot fungi are differentially regulated in response to a variety of environmental signals, including nitrogen and carbon sources, xenobiotics and oxidative stress, among others (Janusz et al., 2013).

4.3. Decay strategies by white-rot wood and humus basidiomycetes

All the above results together suggest two different strategies used by wood and humus white-rot basidiomycetes to colonize and degrade their respective substrates. These strategies would be related to the ability of different regions of the fungal colony to release oxidative enzymes acting on aromatic compounds, including the recalcitrant dyes used in the present study and the lignin polymer in nature (Martínez et al., 2005; Osono, 2007). It was shown that these strategies involve different patterns of oxidoreductase secretion by the fungi assayed that, according to our results, are basically independent on the presence or absence of aromatic compounds (dyes). However, the composition of the culture medium seems to determine the specific enzymes secreted, as demonstrated for the production of peroxidases and laccases able to decolorize dyes that were only detectable when these compounds were present.

It is observed that white-rot wood basidiomycetes release their oxidative enzymes in the area initially colonized by the fungal mycelium. Such behavior has been described for the white-rot wood fungus *P. chrysosporium* growing on dye plates (Moukha et al., 1993), where ligninolytic peroxidases are secreted by secondary hyphae produced in the center of the colony coinciding with the dye decolorization area. We suggest that the decolorization pattern produced by white-rot wood basidiomycetes on agar plates (Fig. 1B, C and E) would be related to their strategy for substrate colonization and degradation in nature. Wood constitutes a hardly-colonized substrate where fungal colonies have physical and nutritional limitations to growth and extend their action. In this context, the production of ligninolytic enzymes seems to be more active in secondary hyphae located at the center of the colony, with a limited diffusion to the outer region and the surrounding medium (Figs. 2 and 4).

On the contrary, white-rot humus basidiomycetes efficiently secrete peroxidases and laccases from young hyphae located at the periphery of the colony (Figs. 1G, 3 and 5B). It has been observed that the secreted oxidoreductases subsequently diffuse in the medium acting on the synthetic dyes in the same way as they would act on leaf litter lignin in nature, allowing the fungi to colonize the soil. Thus, the decolorization pattern observed in white-rot humus basidiomycetes – i.e. outside of the limit of colony – would be related to the nature of the decayed substrate. At the contrary of wood, in this case the substrate is constituted by a whole of unrestricted elements (intermixed dead leaves), and all of this entire system provides a habitat for fungal colonization. Under these conditions, the ligninolytic system would be more active in environment exploring by young hyphae, and could involve the secretion and diffusion of ligninolytic enzymes for degradation of lignin and other aromatic compounds in dead leaves.

5. Conclusions

The differential dye decolorization and enzyme expression/secretion patterns observed suggest that differences in lignocellulose composition and structure could play an important role in wood and humus white-rot (ligninolytic) basidiomycetes diversity. This would include divergent strategies to colonize and degrade wood and leaf-litter by white-rot basidiomycetes, under natural conditions. Such strategies would be related to the metabolic state of the active mycelium, the production of ligninolytic oxidoreductases and their eventual diffusion in the environment, as suggested by the different dye decolorization and enzyme secretion patterns observed in plate cultures of wood and humus fungi. This hypothesis should be corroborated by laboratory studies using more natural (sawdust and leaf litter) growth media. In addition, further investigations aimed to determine the factors regulating oxidoreductase expression and diffusion (or immobilization) are required to explain the differences observed between the two ecophysiological groups of fungi here studied.

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