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

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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, 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,

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).

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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; Westermark 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 H_2O) 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.

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