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Differential regulation of laccase gene expression in *Coriolopsis rigida* LPSC No. 232

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ABSTRACT

Two laccase isoenzyme genes (*lcc2* and *lcc3*) from the white-rot fungus *Coriolopsis rigida* were cloned, and together with the previously described *lcc1*, their transcript levels were analysed by Quantitative RT-PCR in order to study their expression patterns under a range of putative inducers (Cu^{2+}, Mn^{2+}, Fe^{3+}, 2,6-dimethoxy-1,4-benzoquinone, H_2O_2, caffeine, amphotericin B and syringic acid). The highest induction was observed for *lcc1* in presence of copper, and thus, a kinetic study was performed to analyze its effect on temporary *lcc1* gene expression. Our results showed that upregulation due to copper was linked to growth stage, being highest during the trophophase and decreasing during the idiophase. Amphotericin B increased levels of transcripts of *lcc1* and *lcc2*, syringic acid upregulated *lcc1* and *lcc3* and 2,6-dimethoxy-1,4-benzoquinone induced *lcc2* and *lcc3*. Possible reasons for why laccase genes from *C. rigida* are differentially regulated at the transcriptional level are discussed.

Introduction

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are phenol-oxidases belonging to the group of copper containing enzymes, known as blue oxidases (Messerschmidt & Huber 1990). They catalyze the oxidation of a great variety of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor, and thus participating in the production of active oxygen species (Guillén *et al.* 2000) and in the degradation and transformation of lignin and soluble phenols as well as other xenobiotics (Paszczynski & Crawford 1995; Watanabe 2001; Asgher *et al.* 2008). Due to their action over a broad range of substrates, laccases have been widely studied for their potential use in several industrial applications, including pulp bleaching in paper industry, dye decolourisation, detoxification of environmental pollutants and revalorization of wastes and wastewaters (Mayer & Staples 2002; Saparrat *et al.* 2002; Jurado *et al.* 2009). Though laccases are widely distributed among plants, insects and some bacterial species, white-rot fungi have been considered as model organisms for their study and production (Martínez *et al.* 2005; Alcalde *et al.* 2007). In most of fungi, laccases have been found to be encoded by multigene

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families (Valderrama *et al.* 2003). This gene redundancy, which usually involves differences in their physico-chemical and kinetic properties, regulatory mechanisms and localization (Crowe & Olsson 2001; Missall *et al.* 2005), suggest differential physiological roles such as ones related to nutrition, morphogenesis and inter-reaction. Although several studies have analysed the effects of culture parameters on laccase production, specially in order to enhance the yields for industrial applications, there is still some lack of knowledge about the regulation of laccase genes at the molecular level.

Coriolopsis rigida is a white-rot fungus that in the last two decades is being extensively studied for its ability to degrade lignin and several hazardous aromatic pollutants as well as to detoxify and revalorize organic wastes (Sampedro *et al.* 2004; Gómez *et al.* 2006; Saparrat *et al.* 2010). On several *in-vitro* culture conditions, this fungus secretes two laccases as the only ligninolytic enzyme component, which has been characterized in relation to several physico-chemical and catalytic properties (Saparrat *et al.* 2002). Recently it has been reported that these two laccases, encoded by *lcc1* gene, are directly involved in the transformation of aromatic compounds from a solid waste generated during the olive oil production (Díaz *et al.* 2010). The study of transcriptional regulation of this gene as well as other laccase genes in *C. rigida* is important to get a better insight into the oxidative system of this fungus and the physiological roles in which different laccases can be involved. Therefore, the objectives of this research were to identify laccase gene sequences in *C. rigida* and to investigate their gene expression by quantitative RT-PCR (qRT-PCR) under the effect of potentially laccase-inducing compounds. We also performed a kinetic study to analyze the effect of Cu^{2+} on the temporary laccase synthesis.

Materials and methods

Fungal material

Coriolopsis rigida strain LPSC No. 232 (Culture collection of the La Plata Spegazzini Institute = Spanish Type Culture Collection, CECT, no 20449), isolated from decaying wood collected in a subtropical Argentine rain forest (Ibáñez 1998), was maintained on a potato dextrose agar medium (Scharlau Chemie, Barcelona, Spain) at 4 °C.

Preparation of genomic DNA and PCR amplification

Genomic DNA was extracted from mycelial pellets collected from a glucose–peptone liquid medium (Saparrat *et al.* 2002) using Genomix DNA extraction kit (Talent, Italy), according to manufacturer's instructions.

Degenerate primers Pcu_1 (5′ CAYTGGCAYGGNTTYTTYCA 3′) and Pcu_4 (5′ TGRAARTCDATRTGRCARTG 3′), based on the conserved sequences of the copper-binding regions I (HWGGFFQ) and IV (HCHIDFH) and previously described (Hong *et al.* 2007), were used to amplify laccase sequences from *Coriolopsis rigida*.

PCR was performed in 1× PCR amplification buffer (Applied Biosystems) with 1 mM $MgCl_2$ (Applied Biosystems), 1 μM of each primer, 50 μM of each deoxynucleoside triphosphate (Promega), 0.2 μg of DNA template and 1.2 U of Taq DNA polymerase (Applied Biosystems) in a final volume of 50 μL, using a GeneAmp PCR System 2400 (PerkinElmer). Cycling parameters were 95 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 40 s and 72 °C for 1 min and the final extension at 72 °C for 10 min. Control reactions lacking template DNA were performed in parallel. Amplified fragments were visualized on 1 % agarose gels stained with ethidium bromide. PCR products were run in 1 % agarose gel and subsequently cut out, purified by a DNA gel extraction kit (Gene-clean, Q-BIOgene) and then inserted into pGEM-T easy cloning vector (Promega). After transformation of the recombinant vectors into the *Escherichia coli* DH5α strain, clones containing the inserted fragments were screened by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems).

Incubation of Coriolopsis rigida *mycelium with putative laccase inducers*

A mycelial suspension (1 %, v v^{-1}) was inoculated on a glucose–peptone liquid medium with agitation (150 rpm) at 28 °C (Saparrat *et al.* 2002). After 5 d of incubation, mycelial pellets were separated by filtration, washed with sterilized distilled water and 164 ± 12 mg (dry mass) was transferred to flasks containing 50 mL of 50 mM potassium phosphate buffer, pH 5.0, supplemented with the following potential laccase gene inducers: 300 μM Cu^{2+} (as $CuSO_4$ $5H_2O$), 150 μM Mn^{2+} (as $MnSO_4$ H_2O), 100 μM Fe^{3+} (as $FeCl_3$ $6H_2O$), 500 μM 2,6-dimethoxy-1,4-benzoquinone (DQ), 500 μM DQ + 100 μM Fe^{3+}, 500 μM H_2O_2, 5 mM caffeine, 1.5 μM amphotericin B and 1 mM syringic acid. Flasks were then incubated at 28 °C and 150 rpm for 15 h. Mycelium incubated in buffer under the same conditions but with no addition of inducers was used as control. Three replicates were used per treatment. After incubation, mycelia were separated from the buffer by filtration, and divided in two fractions: one used for RNA extraction and the other one to obtain a cell-free extract where laccase activity (considered as mycelium-associated activity) and proteins were measured as described below. The extract was prepared according to Günther *et al.* (1998) but with no addition of reduced glutathione in the extraction buffer. Extracellular laccase activity and proteins were also measured in the incubation liquids as described below.

Preparation of total RNA and reverse transcription

Mycelium pellets incubated for 15 h in control buffer or in the presence of several putative laccase inducers were washed with sterilized distilled water, and total RNA was isolated using Ultraspec RNA kit (Biotecx Laboratories, Inc.) and treated with DNAse I using the 'Deoxyribonuclease I, Amplification Grade' (Invitrogen, UK) to remove the DNA contamination from the samples. First strand complementary DNA (cDNA) was synthesized using the 'GeneAmp Gold RNA PCR Reagent Kit' (Applied Biosystems, USA). This cDNA was used as template in PCR reactions described above.

Quantitative RT-PCR

The sequences amplified by PCR using degenerate primers for the conserved sequences of the copper-binding regions and

corresponding to fragments of genes encoding putative laccases, were aligned by Clustal method using Dnastar (Lasergene, Madison, WI, USA). Primer pairs for qRT-PCR assays were designed for each gene on the basis of this DNA alignment (Table 1) using the Primer Express® software (Applied Biosystems, Foster City, CA). In the case of primers used to amplify 18S gene (the endogenous control gene used to normalize the results), we used the forward NS3 primer previously described (White *et al.* 1990) with a new reverse primer NS4Q designed in this work.

qRT-PCR was used to amplify the three sequences obtained (*lcc1*, *lcc2*, *lcc3*) and 18S cDNA from *C. rigida* using the primers described in Table 1. qRT-PCRs were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems). The PCR thermal cycling conditions for the *lcc1* gene were as follows: an initial step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s, followed by a denaturation step to check the absence of unspecific products or primer dimmers. The same program was used for the other genes except the annealing temperature: 52 °C (*lcc2*), 54 °C (*lcc3*) and 60 °C (18S). SYBR green PCR master mix (Applied Biosystems) was used as the reaction mixture, with the addition of 2.6 μl of sterile Milli-Q water, 1.2 μl of each primer (5 μM), and 5 μl of template cDNA, in a final volume of 20 μl. In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each sample was amplified twice in every experiment.

The results were normalized using the 18S cDNA amplifications run on the same plate. The PCR efficiencies for all the genes were measured by performing a 10-fold serial dilution of positive control template to generate a standard curve, and by plotting the C_T (C_T indicates the cycle in which a target sequence is first detected), as a function of $\log_{10}$ of template. Quantitation is relative to the control gene by subtracting the C_T of the control gene from the C_T of the gene of interest. C_T values were transformed to $\log_2$ (due to the doubling function of PCR) to generate the relative expression levels.

Effect of Cu^{2+} on time course of extracellular laccase activity, growth and lcc1 *gene expression*

The effect of Cu^{2+} on time course of fungal growth, extracellular laccase activity and relative induction of the *lcc1* gene was also analysed. The fungus was grown at 150 rpm and 28 ± 1.5 °C for 15 d on basal medium both plain and supplemented with 300 μM Cu^{2+}. Six replicate cultures were carried out for each treatment. Cultures were filtered and while mycelia from three replicates were used for RNA extraction, the other ones were used for estimating fungal growth by measuring dry biomass. Aliquots of culture supernatants were analysed for laccase activity and proteins as described below.

Table 1 – Oligonucleotide primer sequences used for qRT-PCR.

Gene specificity	Primer name	Primer sequence
lcc1	LCC1-F	5′ GCACGTTGATGATGAATCC 3′
	LCC1-R	5′ CGGTAGCGCTTGCCCTTC 3′
lcc2	LCC2-F	5′ CAACTTCAACTTCACCATC 3′
	LCC2-R	5′ CTCGACATTGACCCATC 3′
lcc3	LCC3-F	5′ CAGGGTCACACGATGAAC 3′
	LCC3-R	5′ CAAGCGTCTGGATGCTG 3′
18S	NS3	5′ GCAAGTCTGGTGCCAGCAGCC 3′
	NS4Q-R	5′ CCGCCCGGCCCAGGTCTG 3′

Enzymatic assays and analysis of protein

Laccase activity was determined by measuring the oxidation of a 5 mM 2,6-dimethoxyphenol (DMP) solution to coerulignone ($\varepsilon_{469} = 27\,500\ M^{-1}\,cm^{-1}$) in 100 mM sodium acetate buffer, pH 5.0. Results were expressed in international enzymatic units (micromoles per minute). Proteins were determined according to Bradford method, using bovine albumin as standard and Bio-Rad kit assay.

Statistical analysis

The data were analysed by a one-way analysis of variance (ANOVA) and means were contrasted by the Least Significant Difference (LSD) test at $p \leq 0.05$ using the SPSS 17.0 software for Windows.

Results

Isolation and characterization of laccase gene fragments

Genomic DNA from *Coriolopsis rigida* was used for PCR amplification using primers based on conserved fungal laccase sequences of the copper-binding regions I and IV (Hong *et al.* 2007). Three single bands were obtained, and they were cloned and sequenced. One of the sequences was unambiguously identified (100 % identity) as the previously reported *lcc1* gene (Díaz *et al.* 2010). The other two sequences were designed as *lcc2* and *lcc3* and deposited on GenBank database under numbers of accession HM461990 and HM461991. These partial sequences, which correspond to the gene region ranging from copper-binding regions I and IV, were compared against databases using TBLASTX 2.2.13 algorithm and showed, respectively, the highest identities with *lac3* (92 %) and *lac2* (83 %) from *Trametes versicolor* sp. C30 (having the accession numbers AY397783 and AF491760, respectively). The deduced amino acid sequences of *lcc1*, *lcc2* and *lcc3* from *C. rigida* matched 91, 91 and 92 % in identity, respectively, to laccases of *T. versicolor* sp. C30 LAC1 (high redox potential laccase), LAC3 and LAC2 (low redox potential laccases) (accession numbers AAM10738, AAR00925 and AAM66348, respectively). The degree of identity among deduced laccase aminoacidic sequences from *C. rigida* was 76.6 % (*lcc2* and *lcc3*), 69 % (*lcc1* and *lcc2*) and 68.3 % (*lcc2* and *lcc3*). The deduced amino acid sequences contain the four copper domains, which are highly conserved in laccases, as well as the His-X-His pattern repeat involved in the coordination of the trinuclear type-2/type-3 copper site.

Development of qRT-PCR protocols

The efficiency of amplification was of 102.6 % for *lcc1*, 97.9 % for *lcc2*, 96.4 % for *lcc3* and 94.6 % for 18S ribosomal gene, being

the slope of the standard curves of −3.361, −3.374, −3.313 and −3.359, respectively. Dimmers and unspecific amplification products were not detected.

Effect of several putative laccase inducers on extracellular and mycelium-associated laccase activity and laccase gene expression

Chemical inducers added to the incubation buffer did not affect the morphology or other characteristics of the mycelium, except in the case of mycelium incubated with syringic acid, which induced the development of dark pigments on the mycelium and in the incubation liquid. In addition to this, the intense yellowish colour of the DQ-containing buffer (either alone or in the presence of Fe^{3+}) disappeared after incubation with *Coriolopsis rigida*. Fig 1 shows the levels of extracellular and mycelium-associated laccase activity (mU mg^{-1} protein) after 15 h of incubation. Amphotericin B and Cu^{2+} induced both extracellular and mycelium-associated laccase activity compared to the controls ($p \leq 0.05$), but while laccase activity was higher in the extracellular fraction in the case of amphotericin B, in the presence of Cu^{2+} the highest activity levels were found associated to mycelium. DQ + Fe^{3+} and syringic acid caused a slight increase in extracellular laccase activity ($p \leq 0.05$) whereas the addition of Mn^{2+}, Fe^{3+}, DQ, H_2O_2 or caffeine did not alter laccase activity at all.

Fig 2 shows the relative expression of *lcc1*, *lcc2* and *lcc3* after 15 h of incubation in response to several putative laccase inducers. When laccase gene upregulation occurred, the highest levels of transcripts were detected for *lcc1* (ranging from 8.5 to 22.3-fold), while *lcc2* and *lcc3* increased their expression only 1.9 to 4.4-fold ($p \leq 0.05$). Amphotericin B, syringic acid and Cu^{2+} triggered transcription of *lcc1* ($p \leq 0.05$), being Cu^{2+} the treatment that caused the highest induction. While amphotericin B and syringic acid induced the expression of either *lcc2* or *lcc3* respectively, DQ triggered the expression of both genes ($p \leq 0.05$). The rest of the treatments did not affect the expression of any of the laccase genes analysed.

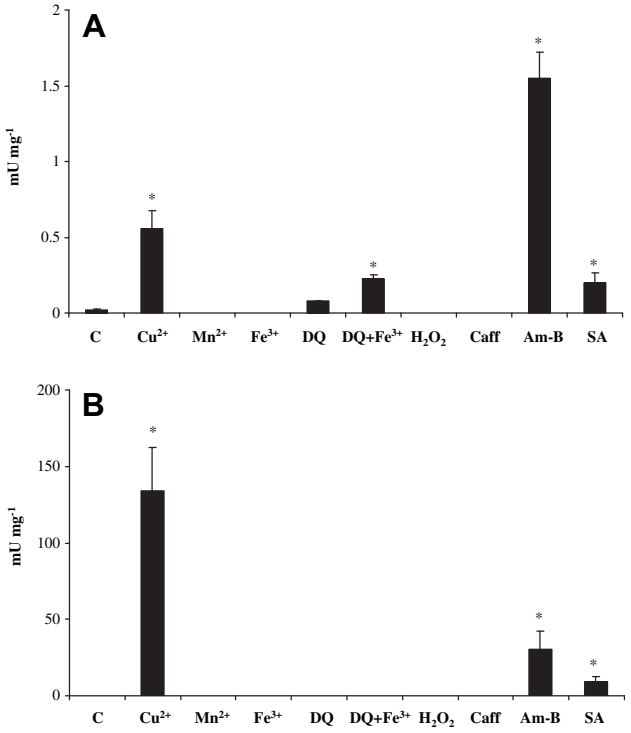

Fig 1 – Extracellular (A) and mycelium-associated (B) *C. rigida* laccase activity (mU mg^{-1} protein), incubated under different potential inducers. C: control, Caff: caffeine, DQ: 2,6-dimethoxy-1,4-benzoquinone, Am-B: amphotericin B, SA: syringic acid. Values are means of three replicates. Error bars correspond to standard deviation. Bars with asterisk are significantly different ($p \leq 0.05$).

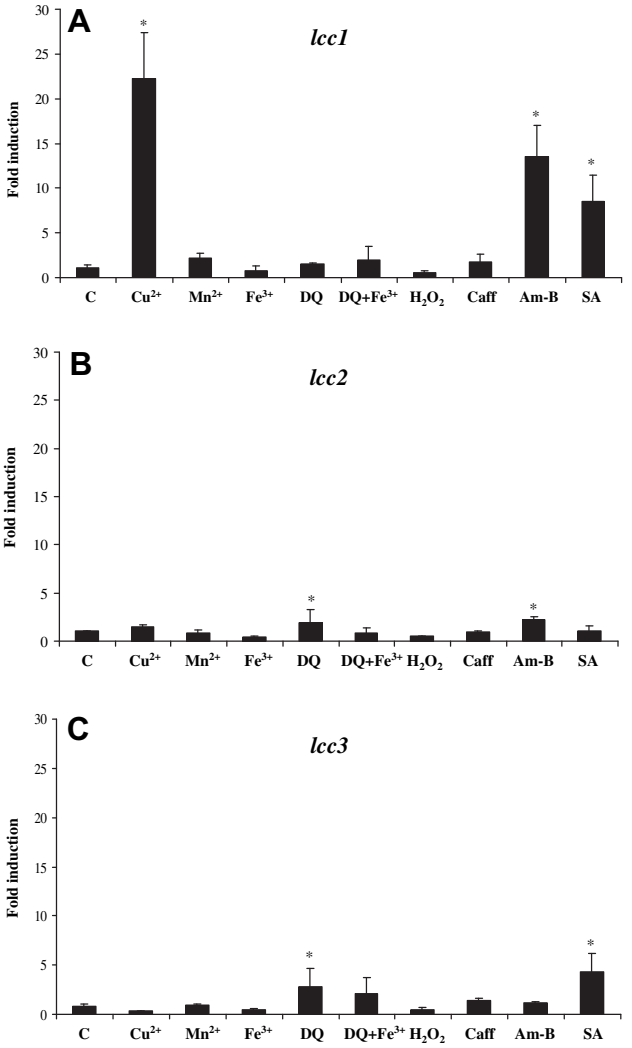

Fig 2 – Relative induction of *C. rigida* laccase genes: *lcc1* (A), *lcc2* (B) and *lcc3* (C) under different potential inducers. The values represent the number of times each gene is expressed in the experiment compared to control treatment (set at 1.00). The results are average of three independent replicates. Error bars correspond to standard deviation. Bars with asterisk are significantly different ($p \leq 0.05$).

Effect of Cu^{2+} on time course of mycelial biomass, extracellular laccase activity and lcc1 *gene expression*

We analysed the effect of addition of Cu^{2+} to the basal medium on time course of biomass, extracellular laccase activity and *lcc1* gene expression (Fig 3). On the basal medium, fungal biomass increased until 10 d after inoculation, thereafter growth remained in a plateau. The addition of Cu^{2+} to the basal medium induced fungal growth in a different way, since the highest biomass was detected at 5 d. Low levels of extracellular laccase activity (<4 mU mL^{-1}) were detected on basal medium after 10 and 15 d. However Cu^{2+} strongly induced laccase activity ($p \leq 0.05$), that increased along the incubation period. Regarding expression of *lcc1*, while the level of transcripts increased concomitantly with fungal growth on basal medium, in the presence of Cu^{2+} high levels of transcripts (271.9-fold) ($p \leq 0.05$) were detected early in the experiment decreasing thereafter concomitantly with growth.

Discussion

The laccase producing fungi may synthesize a number of isoenzymes encoded by multigene families, some constitutively expressed and others in response to regulatory signals (Missall *et al.* 2005; Cañero & Roncero 2008). In the case of *Coriolopsis rigida*, two laccase isoenzymes encoded by *lcc1* gene have been the only ligninolytic enzymatic component found in a glucose–peptone medium or in presence of wastes from olive oil production (Saparrat *et al.* 2002; Díaz *et al.* 2010). In this work we identified two new laccase genes, *lcc2* and *lcc3*, whose partial amino acid sequences display a high degree of similarity with the sequences of the extracellular low redox potential laccases LAC3 and LAC2 from *Trametes* sp. strain C30. The amino acid sequence of the previously described *lcc1* displays a high homology with LAC1, a high redox potential laccase also from C30. It has been reported that LAC1 is

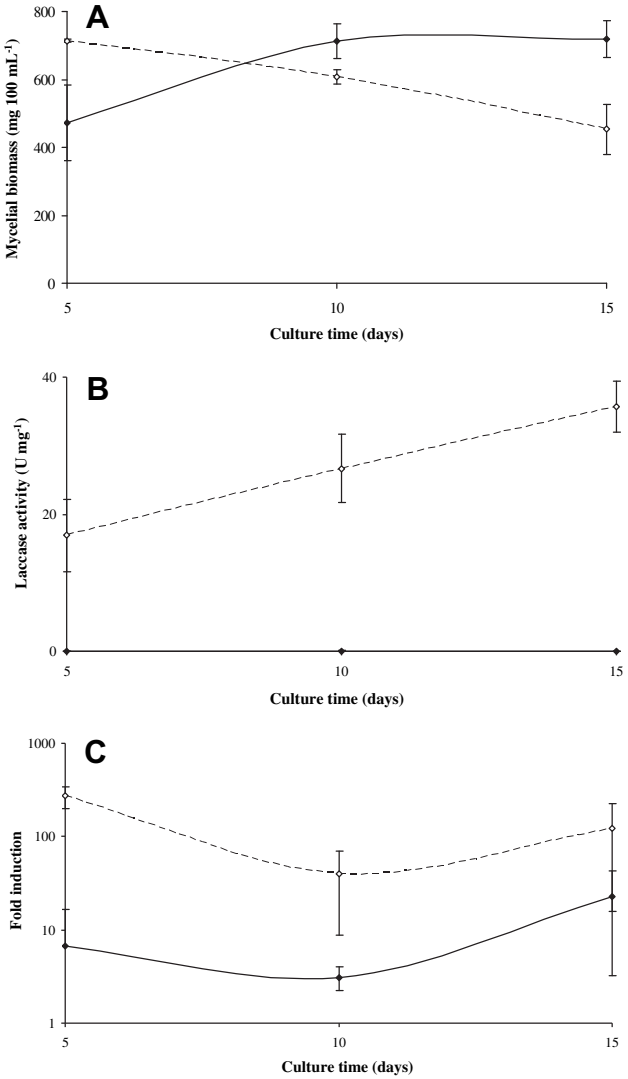

Fig 3 – Time course of mycelial biomass (A), extracellular laccase activity (B) and relative induction of *lcc1* laccase gene (C) from *C. rigida* grown on basal medium (filled diamond) and this medium supplemented with 300 μM $CuSO_4$ (hollow diamond). The values represent the number of times *lcc1* is expressed compared to control (5-d-old mycelium cultured on basal medium, set at 1.00). Values are means of three replicates. Error bars correspond to standard deviation.

a constitutive enzyme which is abundant in culture supernatants, whereas the low redox potential laccases LAC2 and LAC3 need to be induced to be detected, so they can be considered as minor laccase forms (Klonowska *et al.* 2002, 2005). Similarly, in *C. rigida*, *lcc1* showed to be more inducible than *lcc2* and *lcc3* under the putative laccase inducers evaluated. Moreover, *lcc1* encodes the only two laccase isoenzymes detected until now in *C. rigida* cultures (Saparrat *et al.* 2002; Díaz *et al.* 2010). Thus, both the similarity of the amino acid sequence and laccase isoenzyme inducibility suggest that the biology of laccases in *C. rigida* and *Trametes* sp. C30 is similar. It is worth mentioning that both species are closely phylogenetically related, belonging both to *Coriolaceae* family. Until now, C30 is the only organism where simultaneous production of high redox and low redox potential laccases has been reported (Klonowska *et al.* 2002, 2005). However, to our knowledge, gene expression of both low and high redox potential laccases has not yet been analysed in any species. Our data about conditions to induce *lcc2* and *lcc3* in *C. rigida* are a physiological basis for analyzing the production and characterizing the isoenzymes encoded by these genes, especially to confirm their low redox potential. Since both LAC2 and LAC3 are remarkably more catalytically efficient than LAC1 (Klonowska *et al.* 2005), differential inducibility of laccases might be related to their diverse oxidative capability, such as upregulation of high redox potential laccases when the substrate level is abundant and a highly specific induction of laccases with low redox potential when the substrate is low.

The development of qRT-PCR protocols allowed us to evaluate in *C. rigida* the levels of *lcc* gene expression in response to several putative laccases inducers, such as enzyme cofactors (Cu^{2+}) and substrates (syringic acid, DQ), oxidative stress-inducing agents (Mn^{2+}, Fe^{3+}, H_2O_2, DQ + Fe^{3+}) or regulators of signalling pathways involving calcium (amphotericin B) and cyclic adenosine monophosphate (cAMP) (caffeine). Among all the ions tested, only Cu^{2+} increased the transcription levels of *lcc1*, suggesting a metal-responsive induction pathway. It is well known that copper is involved in regulation of laccase at the transcriptional level in a number of basidiomycetes such as *Trametes versicolor* (Collins & Dobson 1997), *Pleurotus ostreatus* (Palmieri *et al.* 2000), *Trametes pubescens* (Galhaup *et al.* 2002), *Pleurotus sajor-caju* (Soden & Dobson 2001) and *Ceriporiopsis subvermispora* (Álvarez *et al.* 2009). However, to date it is not completely understood how copper activates transcription of laccase genes, although response elements similar to those of metallothioneins have been reported in the basidiomycete PM1 (Coll *et al.* 1993) and *C. subvermispora*, (Polanco *et al.* 2002) where an ACE1-like transcription factor is essential for laccase induction with copper addition (Álvarez *et al.* 2009). Another possibility is that laccases might act against oxidative stress to chelate Cu^{2+} ions (Fernández-Larrea & Stahl 1996), given that laccases contain binding sites for copper. In addition, accumulation of the protective pigment melanin, which might be catalyzed by laccases (Eisenman *et al.* 2007) could act against oxidative stress produced by copper. In any case, the fact that Cu^{2+} did not affect the levels of transcription of *lcc2* and *lcc3* would suggest a certain degree of functional specialization for laccase genes in *C. rigida*. Similar findings involving differential transcriptional response to copper have been found in *Volvariella volvacea* (Chen *et al.* 2003) and *P. ostreatus* (Palmieri *et al.* 2000).

Copper caused the highest laccase gene induction in *C. rigida* under the studied conditions. Therefore we evaluated the effect of this ion on temporary *lcc1* gene expression, fungal growth and laccase activity by means of a kinetic study in which copper was added to the basal medium. As previously reported for other white-rot fungi (Mansur *et al.* 1998; Galhaup *et al.* 2002), we found that laccase expression was related to the growth stage since on one hand transcription of *lcc1* was higher during the trophophase than during the idiophase and on the other hand Cu^{2+} enhanced growth and *lcc1* transcription. This might be related to the role that laccases play in lignin degradation so that nutrients are utilized, which might increase during the phase of highest nutritional demands. It appears that the addition of copper resulted in the activation and/or stabilization of laccases, which would explain why although laccase gene transcription was detected in the absence of copper, laccase activity was not observed. This is easily explained because laccases contain four binding domains of copper. In addition, in *P. ostreatus* the presence of copper decreased the activity of an extracellular protease resulting on stabilization of laccase in the crude extracts (Palmieri *et al.* 2000). In any case, the high laccase activity achieved in presence of copper was due mainly to laccase gene upregulation, though a contribution by fungal growth and enzyme activation cannot be ruled out.

Amphotericin B is an antifungal drug which associates with ergosterol from cell walls, altering membrane permeability and eventually leading to a Ca^{2+} influx (Martínez 2007; Takano *et al.* 2009). Amphotericin B induced the expression of *lcc1* and *lcc2*, therefore Ca^{2+} might be involved in the signal transduction pathway of these genes. It has been previously reported in *Cryptococcus neoformans* that amphotericin B induces laccase activity, resulting this on melanin biosynthesis (Eisenman *et al.* 2007) and a subsequent decrease on susceptibility to amphotericin B (Wang & Casadevall 1994). In the case of *C. rigida*, and similarly to oxidative stress produced by Cu^{2+} as mentioned above, the onset of laccase genes could lead also to melanisation of the mycelium as a defense mechanism of fungal cell to counteract the deleterious effect of amphotericin B. It is remarkable that amphotericin B provided the highest extracellular laccase activity, more even than copper, so further investigation to enhance laccases production in *C. rigida* and presumably other laccase producing white-rot fungi could take advantage of this finding.

DQ and syringic acid were of interest in this study because both are laccase substrates. Syringic acid is a phenolic derivative from lignin and DQ is a benzoquinone produced through oxidation of *p*-hydroxyphenyl, guaiacyl, and syringyl units of lignin, which generate hydroxyl radical (·OH) by redox cycling in presence of Fe^{3+} (Gómez-Toribio *et al.* 2009). Incubation of *C. rigida* mycelium on these aromatic compounds resulted in a differential expression of laccase genes. While in the presence of syringic acid, levels of transcription of *lcc1* and *lcc2* increased, the presence of DQ resulted in the induction of *lcc2* and *lcc3*. Conversely, laccase gene expression was not significantly different compared to the control in presence of DQ + Fe^{3+}, and therefore ·OH, although extracellular laccase activity was higher with this combination than only with DQ. This absence of correlation between laccase activity and gene transcription could be indicating the activation of other laccase genes not

yet identified. In any case, it appears that the synthesis of laccases in the presence of DQ + Fe^{3+} was due more to the effect of DQ than to the hydroxyl radicals generated. Although the induction of laccase might be a response to oxidative stress (Ferrari *et al.* 1997; Missall *et al.* 2005; Kim *et al.* 2006), neither H_2O_2 nor DQ + Fe^{3+} produced such effect in *C. rigida*. On the other hand, caffeine, which inhibits phosphodiesterases leading to an increase in cAMP, a secondary messenger that induces laccases in other fungi (Crowe & Olsson 2001; Pukkila-Worley *et al.* 2005), did not upregulate laccase in *C. rigida*.

In conclusion, our results show that *C. rigida* has at least three laccase genes which are differentially regulated at the transcriptional level, being *lcc1* expressed at higher levels than *lcc2* and *lcc3* as a response to several inducers. This differential regulation suggests that laccases from *C. rigida* might respond to different physiological stimuli. The information collected about *lcc2* and *lcc3* upregulation will permit to enhance laccase production in *C. rigida* in order to purify new laccase isoenzymes whose characterization will help to elucidate their biological functions.

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