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Gene expression of *Ceriporiopsis subvermispora* during wood degradation

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Abstract

The biodegradation of wood constituents is currently understood as a multienzymatic process mediated by small molecules. Many cellulolytic and ligninolytic fungi use hydrolytic enzymes that enable them to produce monosaccharides from all of the polysaccharide components of wood. However, when these polysaccharides occur in complexes with lignin, they are resistant to hydrolytic breakdown. Currently, *Ceriporiopsis subvermispora,* one of the most investigated white rot fungi, is heavily used for pretreatment due to its selectively for lignin biodegradation and very low cellulose loss. Thus, the differentially expressed genes of *Ceriporiopsis subvermispora* were identified and studied on *Pinus taeda* and *Eucalytus grandis* cultures from 5 to 60 days. Using the differential display reverse transcription PCR technique (DDRT-PCR), a total of 106 differentially expressed cDNA fragments were identified from mycelia cultures on *P. taeda*. Among these fragments, 65 differential bands were sequenced and identified, and 34 sequences exhibited homologies with known proteins, such as oxidases, dehydrogenases and oxidoreductases. Three of these sequences exhibited similarities to oxalate oxidase, cellobiohydrolase and manganese superoxide dismutase, which are associated with wood biodegradation genes. The q-PCR analysis revealed that the expressions of these genes were associated with the ligninolytic and cellulolytic activities depending on the wood species that was used for fungal growth. These results might provide clues to further our understanding of fungal wood degradation abilities. Although the mechanism remains uncertain, this study identified a set of potentially important genes, including those that hypothetically encode proteins, whose differential expressions provided information that could elucidate the fungal delignification process for biotechnological applications.

Key words: *Ceriporiopsis subvermispora*, wood decay, gene expression, ligninolytic and cellulolytic activities.

Introduction

Several white-rot fungus species have the ability to selectively degrade the lignin components of wood, which has great potential for industrial applications in the processing of agricultural residues, such as corn stover, wheat straw, rice straw, cotton stalks, and woody biomass, to produce cellulose, cellulose-enriched forage for ruminants and biofuel [1-3].

Thus, the main challenge for improving biomass biotechnology is the pretreatment step, which is considered to be one of the most expensive steps in the process of converting lignocelluloses to biofuel [4]. Thus, efforts have been concentrated on the hydrolysis of lignocellulosic material via chemical pretreatment to degrade the lignin, cellulose and hemicellulose [5]. However, this process for removing lignin is often limited due to the lack of selectivity regarding the cell wall components [2, 6]. Therefore, such pretreatments for degrading lignin can be performed with efficient enzymatic hydrolysis, which offers advantages over chemical procedures that include low levels of energy consumption due to the mild process requirements, high sugar yields, and the absence of unwanted waste [7].

Several white rot fungi (*e.g., Ceriporiopsis subvermispora*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*) have been studied for a wide range of biomass feedstock pretreatments [2, 8, 9]. Typically, these fungi have been employed for biopulping, forage upgrading, and soil and wastewater bioremediation due to their abilities to oxidise lignin and lignin-analogous compounds. Thus, such microorganisms are efficient lignin degraders for industrial processes that require biomass delignification [2, 8, 10].

Lignin is a heterogeneous and highly cross-linked macromolecule that is particularly resistant to biological degradation; nevertheless, some fungi have the ability to degrade this macromolecule[11,12]. White-rot fungi are among these microorganisms and an intricate oxidative complex to degrade lignin that is based on oxidative enzymes, such as lignin peroxidases (LiP, EC 1.11.1.14), laccase (Lac, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13), together with low molecular mass mediators [13]. These low molecular mass compounds are diffusible and can initiate wood decay and facilitate the penetration of lignin-degrading enzymes [14].

White rot basidiomycetes differ in their decay morphological patterns. For example, *P. chrysosporium* concomitantly degrades cellulose, hemicellulose, and lignin, and *C. subvermispora* is able to remove lignin prior to cellulose and therefore preserve cellulose during decay [15].

The selective mechanism in *C. subvermispora* lignin degradation appears to involve a manganese-dependent peroxidase and a laccase system for phenolic compound oxidation[13]. This supposition is in agreement with the results of a comparative analysis of the genomes of *C. subvermispora* and *P. chrysosporium,* which revealed that the expression patterns of *C. subvermispora* include increases in oxidoreductases [16]. Therefore, this fungus exhibits potential for use as an efficient enzymatic hydrolysis delignification pretreatment; however, despite the knowledge of the biochemistry and genetics of *C. subvermispora*, several aspects of wood ligninolysis remain unknown. Thus, this report evaluated gene expression in *C. subvermispora* during growth on *Pinus taeda* and *Eucalyptus grandis* wood chips with differential display reverse transcription-PCR (DDRT-PCR) and real-time PCR (qPCR).

Materials and Methods

Wood preparation and culture conditions: Hand-made wood chips (≈ 2.5 cm × 1.5 cm × 0.2 cm) were obtained from 30-year-old trees of *P. taeda* and *E. grandis* (Parque Estadual de Campos de Jordão, SP, Brazil). Prior to the biodegradation assays, these wood chips were immersed in water for 12 h and sterilised in Erlenmeyer flasks (121ºC/15 min).

The *C. subvermispora* SS3 strain was provided by Prof. Dr. André Ferraz (Faculdade de Engenharia Química de Lorena, USP, Brazil). It was grown on 2% malt-extract and 2% agar. After 9 days, 0.2 cm^2 of this culture was transferred to a liquid medium containing 2% malt-extract and incubated for 14 days at 27ºC. Next, 5 g of mycelium were inoculated on wood chips and grown at 27ºC for 5, 10, 15, 20, 40 and 60 days. The culture control was performed using 5 g of mycelium in 2% malt-extract incubated under the same conditions. After each period, the mycelium was collected and stored at -70ºC.

Mycelia growth curves: To estimate the mycelia growth, samples from each culture period were harvested to determine the ergosterol levels using the microwave-assisted extraction method [17]. Briefly, 1 g of milled wood chips were placed in test tubes containing 2.0 ml of 2.0 M NaOH and 8.0 ml methanol and heated in a microwave oven (2450 MHz/620 W/18 s). After cooling (15 min), another 18-s heating cycle was performed. Then, 4.0 ml of 1.0 M HCl and 2 ml of methanol were added and vortexed prior to extraction with 8.0 ml of hexane. The samples were vortexed for 2 min, and hexane fractions were separated and evaporated under a nitrogen stream. All determinations were performed in triplicate. At each time point, the residual material was dissolved in 1.0 ml of methanol, and the ergosterol was analysed with HPLC. The HPLC equipment (Shimadzu LC10AD, Japan) was fitted with a 5-µm ODS2 Spherisorb 150-mm×4.6-mm column (Phase Separation Ltd., UK) with a methanol flow rate of 1.0 ml·min^{-1}. Detection at 282 nm (Shimadzu SPD-10AV detector) with an external calibration was used for ergosterol identification and quantification.

RNA isolation: Mycelium total RNAs from the wood chip cultures were isolated as described by Sokolovsky *et al.*[18] with minor modifications. The mycelia were ground to a powder in liquid nitrogen and lysed with 0.75 ml of lysis buffer (0.6 M NaCl, 10 M EDTA, 100 M Tris-HCl pH 8.0, and 4% SDS). Next, an equal volume of phenol saturated with 0.1 M Tris-HCl, pH 7.4 was added and centrifuged (2000×g/4ºC/10 min). RNA was precipitated from the aqueous phase with 8 M LiCl (0.75×volumes) and incubated overnight at -20ºC. The sample was vortexed and centrifuged (12,000×g /15 min/4ºC), and the pellet was resuspended with 50µl of RNAse-free water. The RNA fungal control culture was isolated with the Perfect RNA kit (Eucariyotic Mini, Eppendorf Scientific Inc., Germany) according to the manufacturer's instructions.

Differential display reverse transcription-PCR: The DDRT-PCR was performed according to the method described by Assmann *et al.*[19] using the arbitrary primers OPJ01, OPJ04, OPJ10 and OPJ12 (Operon Technologies, Alameda CA, USA) and T_{12}NC and T_{12}NG as anchor primers.

The differentially expressed cDNA bands were excised and eluted by incubation at 95ºC for 20 min in 200 µl of deionised water and precipitated with 0.3 M NaCl and 2 volumes of ethanol. After centrifugation (10000×g/20 min), the bands were resuspended in 20 µl of deionised water. To generate the fingerprints, 4 µl of the eluted bands were re-amplified in a 40-µl PCR mixture containing 1 µM of the same primers under the same conditions described previously. The amplified cDNA fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) following the manufacturer's specifications. Clones from each band were sequenced in an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City CA, USA). The results were compared to GenBank sequences using the BlastX function [20].

Quantitative real time-PCR: The housekeeping glyceraldehyde-3-phosphate dehydrogenase (3 g*pd*) gene was used to normalise the quantifications of the differentially expressed genes (Table 1). These reactions were performed in an iCycler (Bio-Rad, Hercules CA, USA) with a final volume of 50 µl containing 25 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™ Life Technologies, Carlsbad CA, USA), 10 µM aliquots of each primer (Table 1) and 100 ng of cDNA under the following conditions: 2 min at 50ºC, denaturation for 2 min at 95ºC, and 40 cycles of 95ºC for 15 s, 59ºC for 15 s and 72ºC for 15 s.

Table 1. Primer sequences used in the q-PCR assays and designed from sequences of bands identified by DDRT-PCR.

Primer	Sequence (5'-3')	Tm (ºC)
gpd -forward	GTGACCTCGTCGTCCGTCT	58.6
gpd - reverse	TCCTTCTCAGCGAAGACGT	58.1
CsOXOX - forward	GGGAGAAGGGACGTGTAAA	56.1
CsOXOX - reverse	TTCTCAACAAAGTGCCCGA	58.3
CsCBH- forward	CGGTACATGGTTCCAGGAG	56.9
CsCBH - reverse	GGTGACATCGCATTGACAA	55.9
CsMnSOD - forward	AAGCCCGAGGGACATAATT	58.0
CsMnSOD - reverse	CACGTCATCCGGTGTCTCC	59.7

Each primer pair was tested for amplification efficiency with q-PCR using template cDNA serial dilutions (100, 10, 1, 0.1 and 0.01 ng μl^{-1}). The results were normalised to the 3 g*pd* gene expression, and the relative expressions of the target genes were calculated using the equation $2^{(Rt-Et)}/2^{(Rn-En)}$, where *Rt* and *Et* are the threshold cycle numbers for the reference genes and the experimental genes, respectively, observed in *Eucalyptus* or *Pinus*; *Rn* is the observed reference gene threshold cycle number in standard conditions (liquid medium culture), and *En* is the threshold cycle number for the experimental gene observed in standard conditions.

Statistical analysis: All results are presented as the mean ± SD, and the statistics were calculated using analyses of variance (ANOVAs) with GraphPad Software version 5.02. Statistical significance was considered at $P \leq 0.05$.

Results and Discussion

Fungal growth: The fungal growth curve analysis indicated that *C. subvermispora* exhibited better colonisation on *E. grandis* than on *P. taeda* (Fig. 1). This finding can be explained based on the lignin structure; i.e., the difference in the syringyl/guaiacyl ratio between hardwood and softwood, which affects wood degradation. Thus, the hardwood *E. grandis* contains both syringyl and guaiacyl lignins, while *P. taeda* (softwood) contains primarily guaiacyl lignins [21, 22]. This growth pattern is consistent with the results of Skyba *et al.*[23] who demonstrated that guaiacyl units are degraded preferentially relative to syringyl moieties by *C. subvermispora* in hardwood.

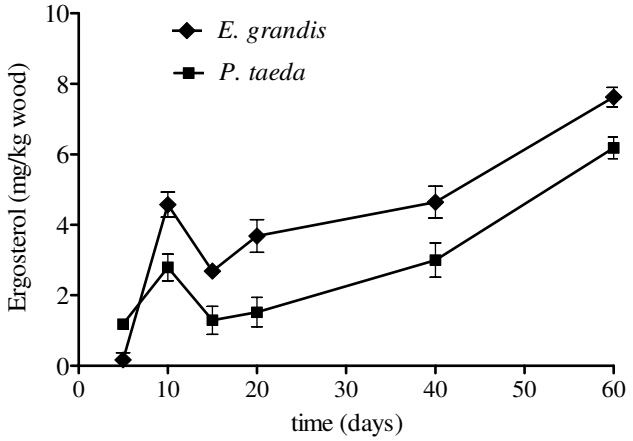

Figure 1. Ergosterol contents of *C. subvermispora* grown on *P. taeda* and *E. grandis* calculated from the dry fungal weights of liquid cultures from 5 to 60 days.

The ergosterol content reflects microorganism viability, and the growth declines on the 15th day on both substrates might be associated with idiophase due to concomitant secondary metabolite synthesis during vegetal biomass loss [17]. The declining growth on 15th day might also be correlated with a lack of nitrogen in the parenchyma cells of the wood chips. Nitrogen is an essential element for fungal growth on wood in the early stages and is typically a highly available nutrient in the parenchyma [24]. Limited nitrogen in the media triggers the production of the fungal ligninolytic enzyme system, which begins the lignin depolymerisation process. With the accumulation of aromatic compounds and reactive oxygen species, the fungal biomass decreases due to an increase in oxidative stress [25]. The *C. subvermispora* ligninolytic enzyme system acts similarly to idiophase due to the low nitrogen and carbon concentrations in the culture medium, which lead to increased MnP, laccase and LiP mRNAs levels [24, 26].

The ergosterol content increased from day 15, which was indicative of fungal growth on the wood chips (Fig. 1). This growth exhibited metabolic activity that was most likely due to wood degradation products that are used in lignin hydrolysis. The degradation products were converted into aerial mycelium, which was observed as a faint white velvet that covered the wood chips. This growth profile observed after 2 weeks of cultivation was the same as that reported by Messner *et al.*[24].

Differential display reverse transcriptase-PCR: The outcome of the DDRT PCR for the total *C. subvermispora* RNA identified 106 cDNA fragments from the fungi grown on *P. taeda*. From these fragments, 65 bands were excised, purified, cloned, sequenced, screened and edited with the BioEdit software. A manual inspection was performed to confirm the quality prior to submitting the sequences to the NCBI database via the BLASTX algorithm. This process revealed 23 differential transcripts that were aligned to hypothetical proteins and eight with no sequence similarities (data not shown). However, 34 sequences exhibited homology with known proteins, such as oxidases, dehydrogenases and oxidoreductases, that are in different organisms, particularly fungi (Table 2).

Regarding DDRT-PCR analyses of the *E. grandis* samples, differentially expressed fragments were not obtained likely due to the presence of phenolic compounds from the wood degradation, which can hinder α-^{33}P-dATP incorporation in the reaction. Similar results have been observed by Salzman *et al.*[27], who analysed the RNA extraction efficiency from plant tissues. Therefore, the *E. grandis* cDNA samples were used only for qPCR.

Thus, three mRNAs with differential expression profiles from *C. subvermispora* grown on *P. taeda* exhibited similarity to oxalate oxidase, cellobiohydrolase and manganese superoxide dismutase, which are genes that are associated with wood biodegradation[15]. These genes were chosen for the assessments of the differential expression on *P. taeda* and *E. grandis* wood chip cultures and in liquid medium using q-PCR.

Quantitative real-time PCR: The q-PCR results for the differential fragment that exhibited similarities of $2e^{-14}$ and $3e^{-12}$ to the oxalate oxidase gene of *Schizophyllum commune* and *C. subvermispora*, respectively, confirmed the differential expression over 60 days of culture. The highest level of expression was observed at day 40 for *E. grandis*. In the *P. taeda* culture, the gene expression began on day 5, reached a maximum at day 10 and had decayed by day 15 (Fig. 2A). Oxalate oxidase (OxOx) is a manganese dependent enzyme that catalyses the oxidation of oxalate to CO_2 and H_2O_2 [28]. Its role in *C. subvermispora* (*Cs*OxOx) is still not well understood; OxOx most likely supplies H_2O_2 to enable the Mn^{+2} to Mn^{+3} oxidation by MnP that promotes lignin degradation [29]. Thus, the *OxOx* expression was significant during the first 15 days of incubation on *P. taeda* chips and in the period between 20 and 60 days on *E. grandis*. An analogous situation has been observed in

Table 2. Sequence homologies of *C. subvermispora* differential transcripts that were identified from the *Pinus taeda* cultures from 5 to 60 days.

Arbitrary primers	Band size (pb)	Fragment name[1]	Sequencing primer[2]	Sequenced bp	Sequence alignment	E-value
	450	Pt51C	+	219	Transferase (*Aspergillus fumigatus*)	9.1
	220	Pt52C	+	213	Fosfatidylinositol glycan, class N (*Homo sapiens*)	3.8
	460	Pt103C	+	485	Oxalate decarboxylase (*Flammulina velutipes)*	2e-12
	450	Pt104AC	+	502	Oxalate decarboxylase (*Flammulina velutipes)*	9e-13
					Oxalate oxidase (*Ceriporiopsis subvermispora*)	3e-12
OPJ01/T12NC	215	Pt151C	+	220	Cytochrome c oxidase polipeptide I (*Hypocrea jecorina*)	1e-16
	517	Pt402C	+	672	Aspartate-ammonia ligase (*Clostridium thermocellum*)	3.4
	410	Pt404C	+	476	Lipoprotein Putative (*Syntrophobacter fumaroxidans*)	5.4
	320	Pt405C	+	325	AroD (*Mycobacterium avium*)	1e-11
	380	Pt603C	+	249	GrpE protein (Hsp-70 cofactor) (*Bacteroides thetaiotaomicron*)	1e-06
OPJ01/T12NG	600	Pt101G	+	329	ABC transporter permease (*Bdellovibrio bacteriovorus*)	2.2
	450	Pt151G	+	340	Plakofiline-3 (*Gallus gallus*).	2.2
	350	Pt101C	+	373	Iron transporter putative (*Chromobacterium violaceum*)	6.4
	250	Pt103C	+	232	Fatty acid oxygenase (*Aspergillus fumigatus*)	1.1
OPJ04/T12NC	700	Pt152C	+	712	Aldo-keto reductase, putative (*Cryptococcus neoformans*)	3e-11
	500	Pt201C	+	496	Gluconate kinase (2-dehydro-3-deoxygluconokinase) (*Bacillus cereus*)	2.6
OPJ04/T12NG	350	Pt105G	+	344	Glycosyltransferase (*Streptococcus agalactiae*)	8.4
	490	Pt602G	+	494	DNA polymerase III putative (*Polaribacter irgensii*)	1e-07
	550	Pt151C	+	186	Cations transporter putative (*Cryptococcus neoformans*)	3.8
	450	Pt153C	+	420	Transcriptional activators putative (*Saccharomyces cerevisiae*)	3.8
OPJ10/T12NC	500	Pt202C	+	168	Cellobiohydrolase (*Coriolus versicolor*)	0.76
	480	Pt204C	+	234	GTPase (*Cryptococcus neoformans*)	0.008
	420	Pt206C	-	476	Manganese superoxide dismutase (*Taiwanofungus camphorata*)	2.9
	440	Pt52G	+	387	Cytochrome b5 reductase (*Zea mays*)	5e-16
					NADH-cytochrome b5 reductase (*Aspergillus nidulans*)	7e-15
	240	Pt154G	+	238	Alpha-1,3-glucanase (*Aspergillus* sp.)	4.2
	700	Pt201G	+	375	Manganese superoxide dismutase (*Taiwanofungus camphorata*)	1e-05
OPJ10/T12NG					Glycosidase (*Cryptococcus neoformans*)	6.8
	350	Pt203G	+	320	OSJNBa0004G10.29 *[Oryza sativa*	6.4
	330	Pt204G	+	428	Gamma-aminobutyrate permease (*Acinetobacter* sp.*)*	6e-43
	850	Pt403G	+	421	Serine/threonine protein kinase (*Frankia* sp.)	2.9
	500	Pt405G	+	404	Tellurium resistant protein (*Azoarcus* sp.)	2.9
	415	Pt406G	+	392	Fibrilarine (*Arabidopsis thaliana*)	0.44
			-	352	NADH: flavin oxidoreductase/NADH oxidase:FAD-dependent pyridine nucleotide-disulphide oxidoreductase (*Chromohalobacter salexigens*)	8.5

[1]Pt = *Pinus taeda*; first two numbers = day of culture; last number = number of isolated band; C = T12NC and G = T12NG. [2]+ = primer T7, - = primer sp6

the growth of *P. chrysosporium* on wood chips at 30 days, which indicates that OxOx might act synergistically in lignin depolymerisation and be indirectly associated with MnP expression[30].

As the differential expression of *OxOx* is substrate-dependent, lignin degradation can be induced by different mechanisms depending on the lignocellulosic composition of the wood. According to Aguiar *et al.* [31], the presence of oxalate during the solid-state fermentation of *P. taeda* wood chips promotes degradation in the early stage of cultures (15 days) and declines until 60 days of culture. These data support our experimental results with respect to *P. taeda,* which expressed *OxOx* in the first 15 days of culture. In contrast, the *OxOX* expression on *E. grandis* was evident in the late stages of degradation, which suggests that the expression of this gene is substrate-dependent [31].

The *MnSOD* q-PCR analysis indicated a similar pattern of expression when *C. subvermispora* was grown on *P. taeda* and reached a high level of expression between the 5th and 10th days; however, on *E. grandis,* the highest level of expression was observed on the 60th day (Fig. 2B). MnSOD catalyses superoxide anion dismutation to H_2O_2 and O_2, and H_2O_2 induces the expression of MnP in white-rot fungi [26]. Therefore, the experimental gene expressions of *MnSOD* and *OxOx* might indicate that these enzymes can promote wood delignification once they are associated with MnP expression [32, 33].

Regarding a fragment that exhibited similarity to cellobiohydrolase II, the q-PCR analysis revealed gene expression pattern that was similar to that obtained for *MnSOD* on *P. taeda* (Fig. 3). The expressions of this fragment on *P. taeda* and *E. grandis* were observed during the mycelial early growth stages (5 to 10 days). However, the cellobiohydrolase expression was significantly higher on *P. taeda* than on *E. grandis*. Additionally, cellobiohydrolase expression was also significantly higher when compared to the expressions of *OxOx* and *MnSOD* on both woods. In fungi, cellobiohydrolases, endoglucanases and β-glucosidases are the enzymes that are required for native cellulose hydrolysis[34]. No significant cellobiohydrolase expression was observed in the submerged *C. subvermispora* culture, but an analysis of the secretome of *C. subvermispora* grown on aspen wood demonstrated cellobiohydrolase expression after the 5th day [35].

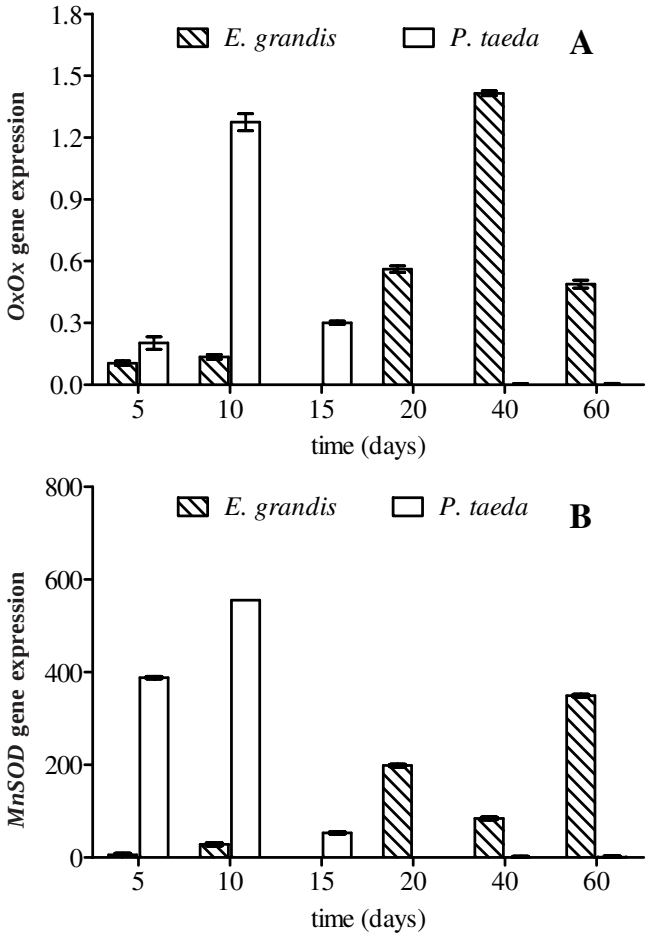

Figure 2. q-PCR expression profiles of the (A) oxalate oxidase (*OxOx*) and (B) *MnSOD* genes of *C. subvermispora* grown on *P. taeda* and *E. grandis* from 5 to 60 days.

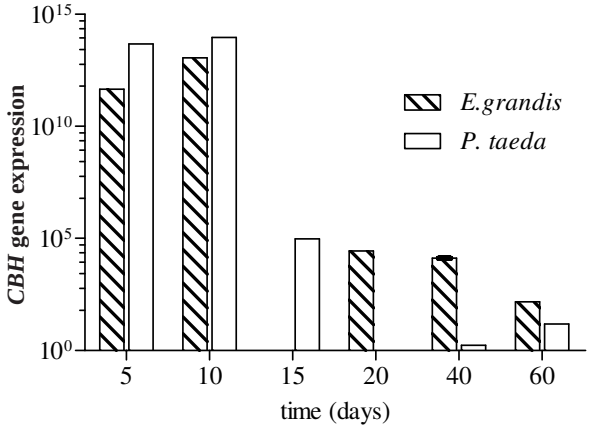

Figure 3. q-PCR expression profiles of the *C. subvermispora* cellobiohydrolase (*CBH*) gene.

Thus, *C. subvermispora* exhibits high cellulolytic activity on pine, which indicates a substrate-dependent expression of cellulolytic genes [34] and corroborates our results.

The three genes of *C. subvermispora* whose expressions were reported in this study were associated with ligninolytic and cellulolytic activities and their differential expression profiles depended on the wood used for fungal growth. During *P. taeda* cultivation, the gene expression over the first 15 days indicated that lignin and cellulose degradation occurred simultaneously. In contrast, the gene expression associated with cellulose degradation in *E. grandis* occurred during the first 15th culture days, and lignin degradation gene expression occurred after this period of growth. These latter findings suggest that the use of *E. grandis* as a substrate might facilitate the separate acquisition of ligninolytic and cellulolytic products during this process.

Conclusions

The identification of induced and repressed genes of *C. subvermispora* grown on different woods might provide clues to further the understanding of fungal wood degradation. Although the mechanisms remain uncertain, this study identified a set of potentially important genes, including those that hypothetically encoded proteins, whose differential expressions provided information that might elucidate the fungal delignification process for biotechnological applications. Thus, microorganisms and their enzymes appear to represent a promising research area for improving the environmental effects and increase the value of biomass. The elucidation of molecular events is crucial to understanding fungal metabolism.

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