

Induction of Extracellular Hydroxyl Radical Production by White-Rot Fungi through Quinone Redox Cycling[∇]

Víctor Gómez-Toribio,¹† Ana B. García-Martín,² María J. Martínez,¹
Ángel T. Martínez,¹ and Francisco Guillén^{2*}

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain,¹
and Departamento de Microbiología y Parasitología, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600,
28871 Alcalá de Henares, Madrid, Spain²

Received 15 September 2008/Accepted 13 April 2009

A simple strategy for the induction of extracellular hydroxyl radical ($\cdot\text{OH}$) production by white-rot fungi is presented. It involves the incubation of mycelium with quinones and Fe^{3+} -EDTA. Succinctly, it is based on the establishment of a quinone redox cycle catalyzed by cell-bound dehydrogenase activities and the ligninolytic enzymes (laccase and peroxidases). The semiquinone intermediate produced by the ligninolytic enzymes drives $\cdot\text{OH}$ production by a Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$). H_2O_2 production, Fe^{3+} reduction, and $\cdot\text{OH}$ generation were initially demonstrated with two *Pleurotus eryngii* mycelia (one producing laccase and versatile peroxidase and the other producing just laccase) and four quinones, 1,4-benzoquinone (BQ), 2-methoxy-1,4-benzoquinone (MBQ), 2,6-dimethoxy-1,4-benzoquinone (DBQ), and 2-methyl-1,4-naphthoquinone (menadione [MD]). In all cases, $\cdot\text{OH}$ radicals were linearly produced, with the highest rate obtained with MD, followed by DBQ, MBQ, and BQ. These rates correlated with both H_2O_2 levels and Fe^{3+} reduction rates observed with the four quinones. Between the two *P. eryngii* mycelia used, the best results were obtained with the one producing only laccase, showing higher $\cdot\text{OH}$ production rates with added purified enzyme. The strategy was then validated in *Bjerkandera adusta*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pycnoporus cinnabarinus*, and *Trametes versicolor*, also showing good correlation between $\cdot\text{OH}$ production rates and the kinds and levels of the ligninolytic enzymes expressed by these fungi. We propose this strategy as a useful tool to study the effects of $\cdot\text{OH}$ radicals on lignin and organopollutant degradation, as well as to improve the bioremediation potential of white-rot fungi.

White-rot fungi are unique in their ability to degrade a wide variety of organopollutants (36, 47), mainly due to the secretion of a low-specificity enzyme system whose natural function is the degradation of lignin (11). Components of this system include laccase and/or one or two types of peroxidase, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (31). Besides acting directly, the ligninolytic enzymes can bring about lignin and pollutant degradation through the generation of low-molecular-weight extracellular oxidants, including (i) Mn^{3+} , (ii) free radicals from some fungal metabolites and lignin depolymerization products (7, 22), and (iii) oxygen free radicals, mainly hydroxyl radicals ($\cdot\text{OH}$) and lipid peroxidation radicals (21). Although $\cdot\text{OH}$ radicals are the strongest oxidants found in cultures of white-rot fungi (1), studies of their involvement in pollutant degradation are scarce. One of the reasons is that the mechanisms proposed for $\cdot\text{OH}$ production still await in vivo validation.

Several potential sources of extracellular $\cdot\text{OH}$ based on the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$) have been postulated for white-rot fungi. In one case, an extracellular fungal glycopeptide has been shown to reduce O_2 and

Fe^{3+} to H_2O_2 and Fe^{2+} (45). Enzymatic sources include cellobiose dehydrogenase, LiP, and laccase. Among these, only cellobiose dehydrogenase is able to directly catalyze the formation of Fenton's reagent (33). The ligninolytic enzymes, however, act as an indirect source of $\cdot\text{OH}$ through the generation of Fe^{3+} and O_2 reductants, such as formate ($\text{CO}_2^{\cdot-}$) and semiquinone ($\text{Q}^{\cdot-}$) radicals. The first time evidence was provided that a ligninolytic enzyme was involved in $\cdot\text{OH}$ production, oxalate was used to generate $\text{CO}_2^{\cdot-}$ in a LiP reaction mediated by veratryl alcohol (4). The proposed mechanism consisted of the following cascade of reactions: production of veratryl alcohol cation radical ($\text{Valc}^{\cdot+}$) by LiP, oxidation of oxalate to $\text{CO}_2^{\cdot-}$ by $\text{Valc}^{\cdot+}$, reduction of O_2 to $\text{O}_2^{\cdot-}$ by $\text{CO}_2^{\cdot-}$, and a superoxide-driven Fenton reaction (Haber-Weiss reaction) in which Fe^{3+} was reduced by $\text{O}_2^{\cdot-}$. The $\cdot\text{OH}$ production mechanism assisted by $\text{Q}^{\cdot-}$ was inferred from the oxidation of 2-methoxy-1,4-benzohydroquinone (MBQH_2) and 2,6-dimethoxy-1,4-benzohydroquinone (DBQH_2) by *Pleurotus eryngii* laccase in the presence of Fe^{3+} -EDTA. The ability of $\text{Q}^{\cdot-}$ radicals to reduce both Fe^{3+} to Fe^{2+} and O_2 to $\text{O}_2^{\cdot-}$, which dismutated to H_2O_2 , was demonstrated (14). In this case, $\cdot\text{OH}$ radicals were generated by a semiquinone-driven Fenton reaction, as $\text{Q}^{\cdot-}$ radicals were the main agents accomplishing Fe^{3+} reduction. The first evidence of the likelihood of this $\cdot\text{OH}$ production mechanism being operative in vivo had been obtained from incubations of *P. eryngii* with 2-methyl-1,4-naphthoquinone (menadione [MD]) and Fe^{3+} -EDTA (15). Extracellular $\cdot\text{OH}$ radicals were produced on a constant basis through quinone redox cycling, consisting of the reduction of MD by a cell-bound quinone reductase (QR) system, followed

* Corresponding author. Mailing address: Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain. Phone: 34918854635. Fax: 34918854663. E-mail: francisco.guillen@uah.es.

† Present address: Varian Iberica S.L., Avda. Pedro Diez 25, 28019 Madrid, Spain.

[∇] Published ahead of print on 17 April 2009.

by the extracellular oxidation of the resulting hydroquinone (MDH₂) to its semiquinone radical (MD^{•-}). The production of extracellular O₂^{•-} and H₂O₂ by *P. eryngii* via redox cycling involving laccase was subsequently confirmed using 1,4-benzoquinone (BQ), 2-methyl-1,4-benzoquinone, and 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone), in addition to MD (16). However, the demonstration of [•]OH production based on the redox cycling of quinones other than MD was still required.

In the present paper, we describe the induction of extracellular [•]OH production by *P. eryngii* upon its incubation with BQ, 2-methoxy-1,4-benzoquinone (MBQ), 2,6-dimethoxy-1,4-benzoquinone (DBQ), and MD in the presence of Fe³⁺-EDTA. The three benzoquinones were selected because they are oxidation products of *p*-hydroxyphenyl, guaiacyl, and syringyl units of lignin (MD was included as a positive control). Along with laccase, the involvement of *P. eryngii* VP in the production of O₂^{•-} and H₂O₂ from hydroquinone oxidation has also been reported (13). Since hydroquinones are substrates of all known ligninolytic enzymes, quinone redox cycling catalysis could involve any of them. Here, we demonstrate [•]OH production by *P. eryngii* under two different culture conditions, leading to the production of laccase or laccase and VP. We also show that quinone redox cycling is widespread among white-rot fungi by using a series of well-studied species that produce different combinations of ligninolytic enzymes.

MATERIALS AND METHODS

Chemicals and enzymes. H₂O₂ (Perhydrol 30%) was obtained from Merck. 2-Deoxyribose, 1,10-phenanthroline, 2-thiobarbituric acid (TBA), and bovine liver catalase (EC 1.11.1.6) were purchased from Sigma. 2,6-Dimethoxyphenol (DMP), 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic (protocatechuic) acid, BQ, DBQ, MD, 1,4-benzohydroquinone (BQH₂), and MBQH₂ were from Aldrich. MBQH₂ was prepared from DBQ by reduction with sodium borohydride (2). MBQ was synthesized by oxidation of MBQH₂ with silver oxide (19). All other chemicals used were of analytical grade.

Laccase isoenzyme I (EC 1.10.3.2) and VP isoenzyme I from *P. eryngii* were produced and purified as previously described by Muñoz et al. (34) and Martínez et al. (32), respectively.

Organisms and culture conditions. *P. eryngii* IJFM A169 (Fungal Culture Collection of the Centro de Investigaciones Biológicas) (= ATCC 90787 and CBS 613.91), *Trametes versicolor* IJFM A136, *Phlebia radiata* IJFM A588 (= CBS 184.83), *Bjerkandera adusta* IJFM A581 (= CBS 595.78), and *Pycnoporus cinnabarinus* IJFM A720 (= CECT 20448; Colección Española de Cultivos Tipo) were maintained at 4°C on 2% malt extract agar. Mycelial pellets were produced at 28°C in shaken (150 rpm) 250-ml conical flasks with 100 ml of a glucose-peptone (GP) medium containing 20 g glucose, 5 g peptone, 2 g yeast extract, 1 g KH₂PO₄, and 0.5 g MgSO₄ · 7 H₂O per liter (25). *P. eryngii* was also cultivated in the presence of 50 μM MnSO₄ (GPMn medium). Inocula were prepared by homogenizing 10-day-old mycelium. The dry weight of the inocula was 0.1 g per 100 ml of medium.

Enzyme activities. Laccase activity was assayed in 100 mM sodium phosphate buffer, pH 5, using 10 mM DMP as a substrate and measuring the production of coerulignone (extinction coefficient at 469 nm [ε₄₆₉] = 27,500 M⁻¹ cm⁻¹, when referring to the DMP concentration) (32). VP and MnP activities were estimated by Mn³⁺-tartrate complex formation (ε₂₃₈ = 6,500 M⁻¹ cm⁻¹) in reaction mixtures containing 100 mM sodium tartrate buffer, pH 5, 100 μM MnSO₄, and 100 μM H₂O₂ (32). LiP activity was assayed in 100 mM tartrate buffer, pH 3, as the oxidation of veratryl alcohol (2 mM) to veratraldehyde (ε₃₁₀ = 9,300 M⁻¹ cm⁻¹) in the presence of 400 μM H₂O₂ (46). These enzymatic assays were performed at room temperature (22 to 25°C).

Mycelium washed with distilled water was used for the determination of cell-bound laccase, VP, and QR activities in *P. eryngii*. Laccase and VP activities were estimated as described above. To minimize underestimates of QR activity, BQ was selected, since laccase activity on BQH₂ has been shown to be quite low (34). QR activity was determined in 50 mM phosphate buffer, pH 5, using 500 μM BQ as a substrate and measuring the production of BQH₂ by high-perfor-

mance liquid chromatography (HPLC). Samples (20 μl) were injected into a Pharmacia system equipped with a Spherisorb S50DS2 column (Hichrom) and a diode array detector. The analyses were carried out at 40°C with a flow rate of 1 ml min⁻¹ and 10 mM phosphoric acid-methanol (80/20) as an eluent. The UV detector operated at 280 nm, and BQH₂ levels were estimated using a standard calibration curve. For these cell-bound analyses of enzymatic activities, appropriate amounts of mycelium were incubated at room temperature with 20-ml substrate solutions in shaken 100-ml conical flasks (150 rpm). Samples were taken at 1-min intervals for 5 min. The mycelium was separated from the liquid by filtration. Absorbance was determined immediately after filtration for laccase and VP activity measurements. The pH of samples used for QR activity determination was lowered to 2 with phosphoric acid, and they were kept frozen at -20°C until they were analyzed. International units of enzyme activity (μmol min⁻¹) were used.

Incubation of fungi with quinones. Ten-day-old mycelial pellets were collected from cultures by filtration, washed three times with distilled water, and resuspended in 50 ml of 20 mM phosphate buffer, pH 5, containing 500 μM BQ, MBQ, DBQ, or MD. The amount of mycelium used in these incubations was 202 ± 14 mg (dry weight). For Fe³⁺ reduction experiments, the complex 100 μM Fe³⁺-110 μM EDTA and 1.5 mM 1,10-phenanthroline were added to the quinone incubation solution. Iron salt (FeCl₃) solutions were made up fresh immediately before use. In [•]OH production experiments, 1,10-phenanthroline was replaced by either 2.8 mM 2-deoxyribose or 1 mM 4-hydroxybenzoic acid, depending on the method used to estimate [•]OH generation (see below). Incubations were performed in 50-ml volumes at 28°C and 150 rpm in 100 ml conical flasks. Samples were periodically removed from three replicate flasks, and the extracellular fluid was separated from the mycelium by filtration. In order to inactivate the ligninolytic enzymes that could be released to the extracellular solution during the experiments, samples were treated in different ways depending on the kind of analysis to be performed. For the analysis of quinone, hydroquinone, protocatechuic acid, and TBA-reactive substances (TBARS), the pH of samples was lowered to 2 with phosphoric acid. For H₂O₂ estimation, samples were heated at 80°C for 20 min (a treatment that does not affect H₂O₂ levels). Other analyses were carried out immediately after the samples were removed.

Analytical techniques. The Somogyi-Nelson method for the determination of reducing sugars was used to estimate glucose concentrations in fungal cultures (41).

Levels of MBQ, DBQ, and their corresponding hydroquinones were determined by HPLC, using standard calibration curves for each compound (17). Samples (20 μl) were injected into a Pharmacia system equipped with a Spherisorb S50DS2 column (Hichrom). The analyses were carried out at 40°C with a flow rate of 1 ml min⁻¹ using 10 mM phosphoric acid-methanol (80/20) as the eluent. The UV detector operated at 254 and 280 nm.

H₂O₂ levels were estimated by measuring the production of O₂ with a Clark-type electrode after the addition of 100 U of catalase per ml (heat-denatured catalase was used in blanks) (17). The amount of H₂O₂ was calculated taking into account the stoichiometry of the catalase reaction (2 H₂O₂:1 O₂). The oxygen electrode was calibrated by the same procedure with known amounts of H₂O₂ from the standard commercial solution, which were calculated spectrophotometrically (ε₂₃₀ = 81 M⁻¹ cm⁻¹).

The production of ferrous ion was measured at 510 nm by the formation of its chelate with 1,10-phenanthroline (ε = 12,110 M⁻¹ cm⁻¹) (4).

Both TBARS production from 2-deoxyribose (20) and conversion of 4-hydroxybenzoic acid into protocatechuic acid (12) were used as procedures to estimate [•]OH production. TBARS were determined as follows. A total of 0.5 ml of 2.8% (wt/vol) trichloroacetic acid and 0.5 ml of 1% (wt/vol) TBA in 50 mM NaOH was added to 1-ml samples and heated for 10 min at 100°C. After the mixture cooled, the absorbance was read at 532 nm against appropriate blanks (18). Protocatechuic acid production was estimated by HPLC as described above for quinones, except a linear 10 mM phosphoric acid/methanol gradient from 0 to 30% methanol in 20 min was used as the mobile phase. The absorbance of the eluate was monitored at 254 nm (14).

Statistical analysis. All the results included in the text and shown in the figures are the means and standard deviations of three replicates (full biological experiments and technical analyses). The least-squares method was used for regression analysis of data.

RESULTS

Following our previous studies of quinone redox cycling (15, 16), incubations of *P. eryngii* with quinones were carried out in buffered solutions (pH 5) and 10-day-old washed pellets. The fungus was grown in GP medium with or without Mn²⁺ in

TABLE 1. Extracellular and cell-bound laccase, VP, and quinone reductase activities present in 10 day-old cultures of *P. eryngii* grown in GP and GPMn media

Medium	Enzyme	Activity	
		Culture liquid (mU ml ⁻¹)	Cell bound (mU mg ⁻¹)
GP	Lac	24 ± 2	8 ± 1
	VP	330 ± 13	4 ± 1
	QR	ND ^a	30 ± 3
GPMn	Lac	57 ± 5	11 ± 2
	VP	0	0
	QR	ND	28 ± 3

^a ND, not determined.

order to obtain pellets producing, respectively, only laccase (Lac-mycelium) or laccase plus VP (LacVP-mycelium) (32). In both cases, maximum growth was reached after 8 to 12 days, coinciding with glucose depletion (10 days). The mycelial dry weights in 10-day-old cultures carried out in GP and GPMn media were 808 ± 54 mg (average of three replicate flasks ± standard deviation). The levels of extracellular and cell-bound laccase, VP, and QR activities in 10-day-old cultures are listed in Table 1. The presence of Mn²⁺ in the culture medium

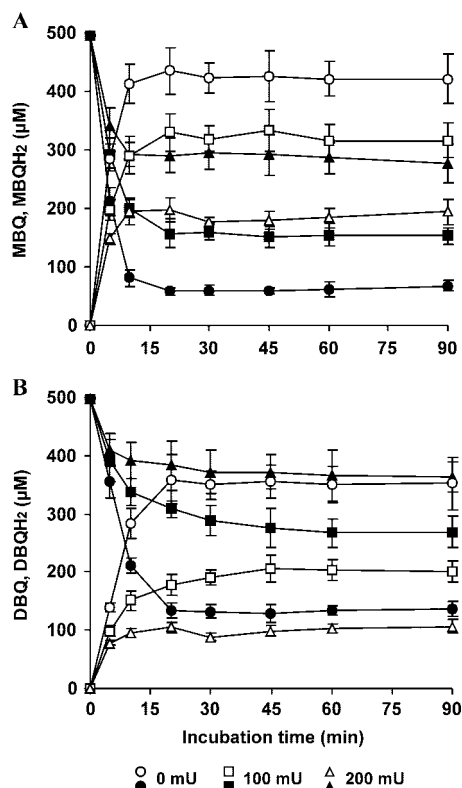


FIG. 1. Time course of MBQ (A) and DBQ (B) reduction by *P. eryngii* in the presence of different amounts of laccase (filled symbols, MBQ and DBQ; open symbols, MBQH₂ and DBQH₂). Incubations were carried out with 10-day-old Lac-mycelium and 500 µM quinones in 50 ml 20 mM phosphate buffer, pH 5, in the absence and presence of 100 and 200 mU ml⁻¹ laccase. The error bars indicate standard deviations.

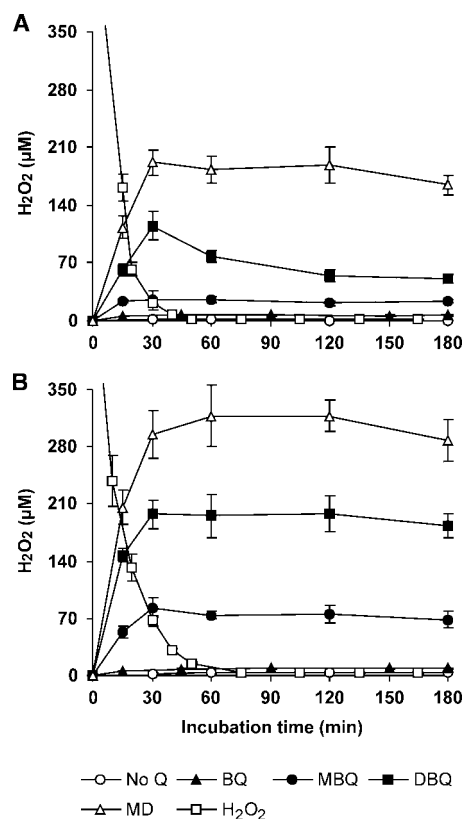


FIG. 2. Effects of quinones on the production of H₂O₂ by LacVP-mycelium (A) and Lac-mycelium (B) from *P. eryngii*. Mycelia were incubated in 50 ml 20 mM phosphate buffer, pH 5, in the absence (No Q) and presence of 500 µM BQ, MBQ, DBQ, or MD. Incubations with 500 µM H₂O₂ were also carried out in the absence of quinones. The error bars indicate standard deviations.

repressed the production of VP (38), increased laccase activity, and had no significant effect on cell-bound QR activity.

As previously reported for BQ and MD (16), MBQ and DBQ were redox cycled when incubated with washed Lac-mycelium (Fig. 1). Reduction of MBQ and DBQ to their corresponding QH₂s was observed during the first 20 min. Then, QH₂/Q molar ratios remained constant until the end of the experiment: 6.8 and 2.6 (average of 30- to 90-min samples) for MBQ and DBQ, respectively. These ratios were the result of equilibrium between QR and laccase activities as confirmed in parallel experiments carried out with added laccase. It was found that when the extracellular laccase activity was raised to 100 and 200 mU ml⁻¹, QH₂/Q ratios decreased, respectively, to 2.1 and 0.6 for MBQ and to 0.7 and 0.3 for DBQ. The lower ratios observed in incubations with DBQ agreed with the higher efficiency of laccase oxidizing DBQH₂ than MBQH₂ (17). These results indicated that oxidation of hydroquinones in the absence of added laccase was the rate-limiting step of MBQ and DBQ redox cycles, and therefore, that the enzyme addition increased the rates of these cycles.

The production of H₂O₂ and reduction of Fe³⁺ by *P. eryngii* upon incubation with BQ, MBQ, DBQ, and MD was evaluated as a requisite for ·OH generation. Figure 2A and B shows the results for H₂O₂ production by LacVP-mycelium and Lac-mycelium, respectively. Regardless of the mycelium used, basal

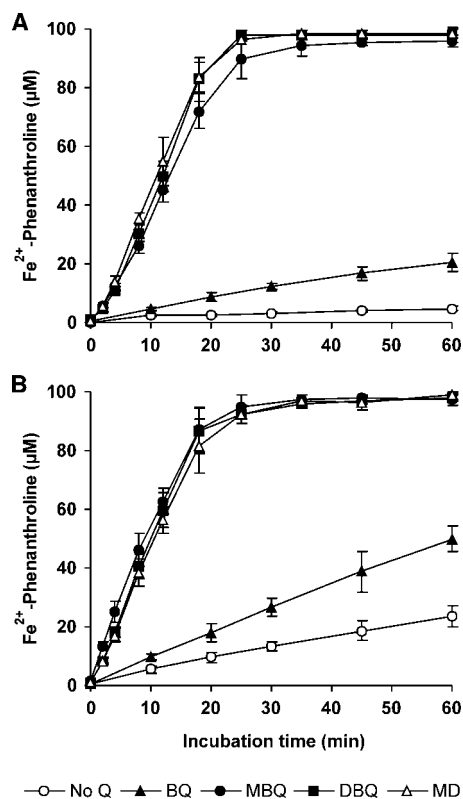


FIG. 3. Effects of quinones on the reduction of chelated Fe^{3+} by LacVP-mycelium (A) and Lac-mycelium (B) from *P. eryngii*. Mycelia were incubated in 50 ml 20 mM phosphate buffer, pH 5, in the absence (No Q) and presence of 500 μM BQ, MBQ, DBQ, or MD, with 100 μM Fe^{3+} -110 μM EDTA and 1.5 mM 1,10-phenanthroline. The error bars indicate standard deviations.

levels of extracellular H_2O_2 observed in 60- to 180-min samples remained around 2 to 3 μM with no quinone added. However, incubations carried out with quinones gradually increased H_2O_2 levels until steady values were attained (after 30 min). The average levels of 60- to 180-min samples increased in the order $\text{BQ} < \text{MBQ} < \text{DBQ} < \text{MD}$ and were lower with LacVP-mycelium (6, 23, 61, and 179 μM , respectively) than with Lac-mycelium (8, 72, 192, and 308 μM , respectively). Figure 2 also shows that when *P. eryngii* mycelia were incubated with 500 μM H_2O_2 , it nearly disappeared from the extracellular liquid in about 60 min. After this time, H_2O_2 levels were similar to those found without quinones (2 to 3 μM). Although this decrease in H_2O_2 levels was observed with both mycelia, indicating that they have one or more systems consuming it, the higher consumption rate observed with LacVP-mycelium suggested the involvement of VP. From these findings, it could be inferred that the steady H_2O_2 levels found in incubations with quinones were the result of equilibrium between the rates of production and consumption, and therefore, that quinone redox cycling was operative during the whole period studied.

On the other hand, the effect of quinones on Fe^{3+} reduction by washed *P. eryngii* mycelia is shown in Fig. 3. Fe^{2+} -phenanthroline complex from Fe^{3+} -EDTA was linearly produced at different rates depending on the mycelium and quinone used. In the absence of quinones, basal iron reduction rates calcu-

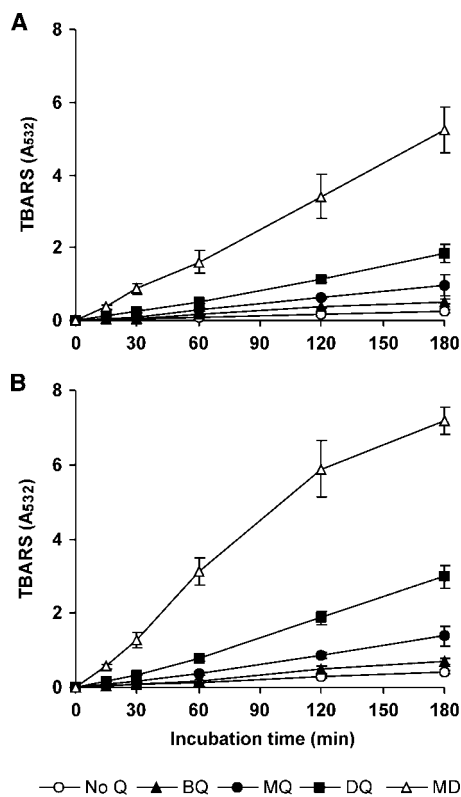


FIG. 4. Hydroxyl radical production by LacVP-mycelium (A) and Lac-mycelium (B) from *P. eryngii* via quinone redox cycling, estimated as TBARS formation from 2-deoxyribose. The incubation mixtures were as described in the legend to Fig. 3, except that 1,10-phenanthroline was replaced by 2.8 mM 2-deoxyribose. The error bars indicate standard deviations.

lated from 0- to 60-min samples were 0.1 and 0.4 $\mu\text{M min}^{-1}$ with LacVP- and Lac-mycelium, respectively. This observation supports the existence of a cell-bound mechanism for iron reduction which did not imply quinone redox cycling. Incubations with BQ raised these rates to 0.4 $\mu\text{M min}^{-1}$ (LacVP-mycelium) and 0.9 $\mu\text{M min}^{-1}$ (Lac-mycelium). A much greater increase was obtained in incubations with MBQ, DBQ, and MD, although no significant differences were found among them: 4.0 $\mu\text{M min}^{-1}$ with LacVP-mycelium and 5.0 $\mu\text{M min}^{-1}$ with Lac-mycelium, as calculated from the linear increase observed in 0- to 12-min samples. The Fe^{3+} was completely reduced and scavenged by phenanthroline with these three quinones in about 25 min.

Following the demonstration of the formation of Fenton's reagent, production of $\cdot\text{OH}$ was studied in incubations of washed mycelia with quinones and Fe^{3+} -EDTA. Depending on the procedure used to detect $\cdot\text{OH}$, the incubation mixtures also contained either 2-deoxyribose or 4-hydroxybenzoic acid for the production of TBARS or protocatechuic acid, respectively. TBARS production was linear during the whole period studied with the four quinones and the two mycelia used (Fig. 4). The TBARS production rates were always higher with Lac-mycelium than with LacVP-mycelium. In both cases, the highest rate was obtained with MD, followed by DBQ, MBQ, and BQ. There existed, therefore, a positive correlation between

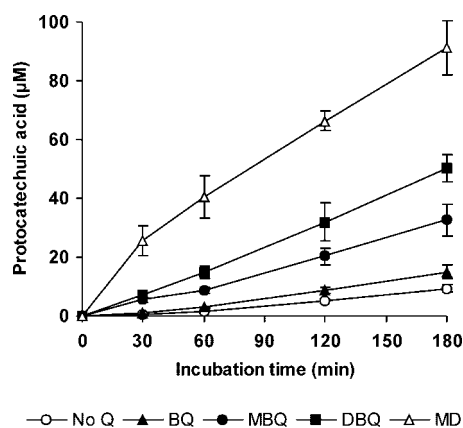


FIG. 5. Hydroxyl radical production by Lac-mycelium from *P. eryngii* via quinone redox cycling, estimated as 4-hydroxybenzoic acid hydroxylation. The incubation mixtures were as described in the legend to Fig. 3, except 1,10-phenanthroline was replaced by 1 mM 4-hydroxybenzoic acid. The error bars indicate standard deviations.

TBARS levels and those of H_2O_2 shown in Fig. 2. Furthermore, taking into account that Fe^{2+} was produced at a rate similar to those of MBQ, DBQ, and MD (Fig. 3), it could be inferred that H_2O_2 was limiting the Fenton reaction during at least MBQ and DBQ redox cycling. TBARS production was also observed in the absence of quinones and the presence of Fe^{3+} -EDTA, but at the lowest rates. This observation is in close agreement with the results in Fig. 3, showing the ability of *P. eryngii* to reduce Fe^{3+} with no Q. In addition to H_2O_2 basal levels generated by the fungus under these conditions (Fig. 2), $\cdot OH$ production could be supported by that derived from ferrous iron autoxidation, followed by the dismutation of the resulting $O_2^{\cdot -}$.

In order to confirm $\cdot OH$ production, the effect of $\cdot OH$ scavengers, such as mannitol and dimethyl sulfoxide (DMSO), on TBARS formation was evaluated. Lac-mycelium was incubated with DBQ, Fe^{3+} -EDTA, and 2-deoxyribose in the absence and presence of 5 mM mannitol or DMSO. The TBARS production rate during the first 120 min decreased from 14.8 $mU A_{532} \text{ min}^{-1}$ (incubation without scavengers) to 7.4 and 2.8 $mU A_{532} \text{ min}^{-1}$ with mannitol and DMSO, respectively. The production of $\cdot OH$ was also confirmed by estimating the hydroxylation of 4-hydroxybenzoic acid during the redox cycling of the four quinones by Lac-mycelium. As shown in Fig. 5, protocatechuic acid was also produced on a constant basis throughout the experiment. Regression analyses of data obtained from 30- to 180-min samples produced the following rates of protocatechuic acid production: 64, 102, 199, 297, and 423 $nM \text{ min}^{-1}$ in incubations with no Q, BQ, MBQ, DBQ, and MD, respectively. The results in Fig. 1 revealed that oxidation of $MBQH_2$ and $DBQH_2$ was the rate-limiting step of the MBQ and DBQ redox cycles. Based on these results, the effects of increasing extracellular laccase and VP activities on $\cdot OH$ production were evaluated. Figure 6 shows TBARS production during the incubation of Lac-mycelium with DBQ, Fe^{3+} -EDTA, 2-deoxyribose, and different amounts of laccase and VP. The addition of 50 and 100 $mU \text{ ml}^{-1}$ laccase enhanced the TBARS production rate from 17 $mU A_{532} \text{ min}^{-1}$ (blank without added enzymes) to 40 and 50 $mU A_{532} \text{ min}^{-1}$, respectively,

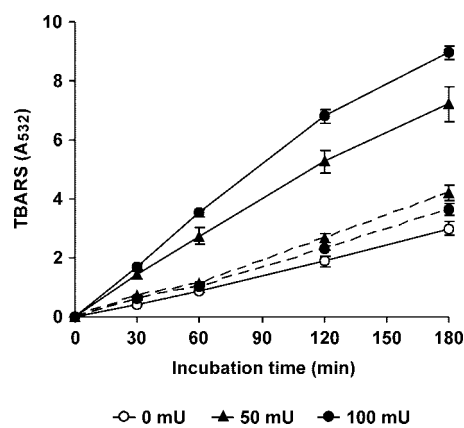


FIG. 6. Effects of laccase and VP on hydroxyl radical (TBARS) production by Lac-mycelium from *P. eryngii* via DBQ redox cycling. The incubations were carried out in 50 ml 20 mM phosphate buffer, pH 5, containing Lac-mycelium, 500 μM DBQ, 100 μM Fe^{3+} -110 μM EDTA, and 2.8 mM 2-deoxyribose, in the absence (0 mU) and presence of 50 and 100 $mU \text{ ml}^{-1}$ laccase (solid lines) and VP (dashed lines). The error bars indicate standard deviations.

as calculated from the results obtained for 0- to 180-min samples. The addition of VP also caused an increase in the TBARS production rate, although much lower and inversely correlated with the amount of the enzyme: 23 and 20 $mU A_{532} \text{ min}^{-1}$ with 50 and 100 $mU \text{ ml}^{-1}$ of added VP, respectively.

A final series of experiments was carried out in order to test the validity of this inducible $\cdot OH$ production mechanism in other white-rot fungi: *B. adusta*, *P. chrysosporium*, *P. radiata*, *P. cinnabarinus*, and *T. versicolor*. Quinone redox cycling experiments were carried out with DBQ and 10-day-old washed mycelia grown in GP medium. The ligninolytic enzymes produced by the fungi under these culture conditions were LiP and a Mn^{2+} -oxidizing peroxidase (VP) by *B. adusta* (30); laccase by *P. cinnabarinus*, *T. versicolor*, and *P. radiata*; and none by *P. chrysosporium* (Table 2). These different enzyme production patterns, including several levels of laccase, allowed us to study the effect of ligninolytic enzymes on $\cdot OH$ production from both qualitative and quantitative points of view. Incubations of the fungi with DBQ were carried out in the presence of Fe^{3+} -EDTA and 2-deoxyribose, and samples were analyzed for TBARS production, as well as for DBQ and $DBQH_2$ levels (Fig. 7). Among the five fungi, *P. chrysosporium* produced TBARS at the lowest rate (2 $mU A_{532} \text{ min}^{-1}$ during a period from 0 to 120 min). Although the fungus showed a good ability to reduce DBQ, it is quite likely that the absence of ligninolytic enzymes limited the production of TBARS. The highest

TABLE 2. Extracellular laccase, MnP/VP, and LiP activities present in 10-day-old cultures of selected white-rot fungi grown in GP medium

Species	Enzyme activity ($mU \text{ ml}^{-1}$)		
	Laccase	MnP/VP	LiP
<i>B. adusta</i>	0	38 \pm 3	192 \pm 15
<i>P. chrysosporium</i>	0	0	0
<i>P. radiata</i>	319 \pm 22	0	0
<i>P. cinnabarinus</i>	19 \pm 1	0	0
<i>T. versicolor</i>	217 \pm 14	0	0

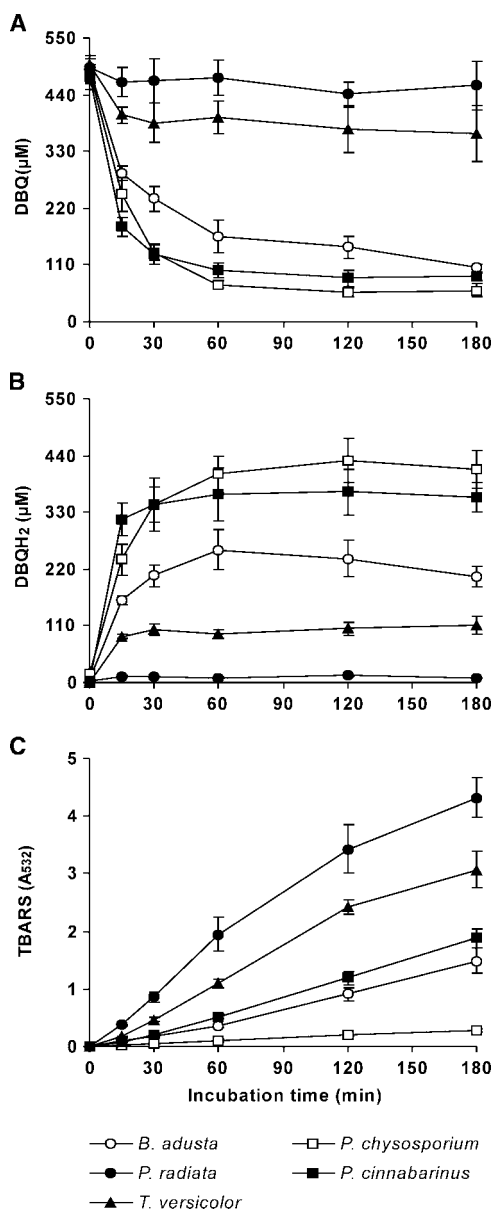


FIG. 7. Hydroxyl radical production by selected white-rot fungi via DBQ redox cycling. Levels of DBQ (A), DBQH₂ (B), and TBARS (C) are shown. The incubation mixtures contained 50 ml 20 mM phosphate buffer, pH 5; 200 ± 12 mg 10-day-old mycelia from *B. adusta*, *P. chrysosporium*, *P. radiata*, *P. cinnabarinus*, and *T. versicolor*; 500 µM DBQ; 100 µM Fe³⁺–110 µM EDTA; and 2.8 mM 2-deoxyribose. TBARS levels were corrected from those found in incubation blanks without DBQ. The error bars indicate standard deviations.

TBARS production rate, estimated during the same period, was obtained with *P. radiata*, followed by *T. versicolor* and *P. cinnabarinus*, i.e., 29, 21, and 10 mU A₅₃₂ min⁻¹, respectively (Fig. 7C). These results were positively correlated with laccase activity levels (Table 2), in agreement with the positive effect caused by the enzyme on TBARS production by *P. eryngii* (Fig. 6). Furthermore, the differences in laccase levels were also reflected in the DBQH₂/DBQ ratios found at system equilibrium (Fig. 7A and B), similar to the results obtained with *P. eryngii* (Fig. 1). Thus, whereas DBQH₂ was the prevalent com-

pound in 60- to 180-min samples from the fungus producing the lowest laccase activity (*P. cinnabarinus*), DBQ was the prevalent compound with the best laccase producers (*P. radiata* and *T. versicolor*). A lower TBARS production rate was observed with *B. adusta* (8 mU A₅₃₂ min⁻¹), producing only ligninolytic peroxidases. However, it was four times higher than that observed with *P. chrysosporium*, indicating the involvement of these enzymes in DBQ redox cycling.

DISCUSSION

Our results provide in vivo validation of the Q^{•-}-assisted mechanism of reactive oxygen species (ROS) production previously demonstrated with purified laccases from *P. eryngii* (14, 17, 34) and *Corioloropsis rigida* (39), VP from *P. eryngii*, and MnP from *Phanerochaete chrysosporium* (13). They also support our earlier finding that quinone redox cycling is a mechanism for the production of extracellular O₂^{•-} and H₂O₂ in *P. eryngii* (16). This mechanism can now be expanded to [•]OH production, a greater number of quinones, and some widely studied white-rot fungi. The production of [•]OH radicals has been inferred from the generation of Fe²⁺ and H₂O₂ (Fenton's reagent), as well as from the conversion of 2-deoxyribose into TBARS and the hydroxylation of 4-hydroxybenzoic acid, with the four parameters highly correlated. A definitive proof confirming [•]OH identity would be required, such as electron spin resonance analysis. Overall, these results enable us to propose the scheme depicted in Fig. 8 as a model for extracellular [•]OH production by white-rot fungi via quinone redox cycling. Although the scheme shows the main reactions involved in ROS production under the incubation conditions used in the present study, some steps of the process may present several alternatives (described below). As can be seen, two redox cycles are shown, one for the three benzoquinones (Fig. 8A) and the other for the naphthoquinone (Fig. 8B). Common reactions involved in the generation of Fenton's reagent in both cycles (reactions 4a, 5a to c, and 6) are shown only in Fig. 8A in order to avoid a crowded scheme. Figure 8B includes a distinctive reaction of MD redox cycling, i.e., propagation of MDH₂ oxidation by O₂^{•-} (reaction 4b), which, as discussed below, was probably the factor contributing most to the production of the highest levels of H₂O₂ and [•]OH radicals with this quinone (Fig. 2 and 4, respectively). With the exception of the enzymatic oxidation of QH₂, a similar quinone redox cycling process has been described in brown-rot fungi. These fungi produce several methoxyhydroquinones that can be chemically oxidized by Fe³⁺ (24). The mechanisms by which these fungi produce extracellular [•]OH have been widely studied, since these radicals are considered the main agents causing the rapid cellulose depolymerization characteristic of brown rot (3). Thus, the involvement of QH₂ in ROS production by brown-rot fungi has been evidenced not only under defined liquid incubation conditions, but also on cellulose and wood (8, 44). Quinone redox cycling in other biological systems has been extensively studied and described as an intracellular process consisting of the enzymatic one-electron reduction of Q, followed by the autoxidation of the resulting Q^{•-} (23). However, quinone redox cycling in white- and brown-rot fungi presents the distinctive characteristic of producing ROS in the extracellular environ-

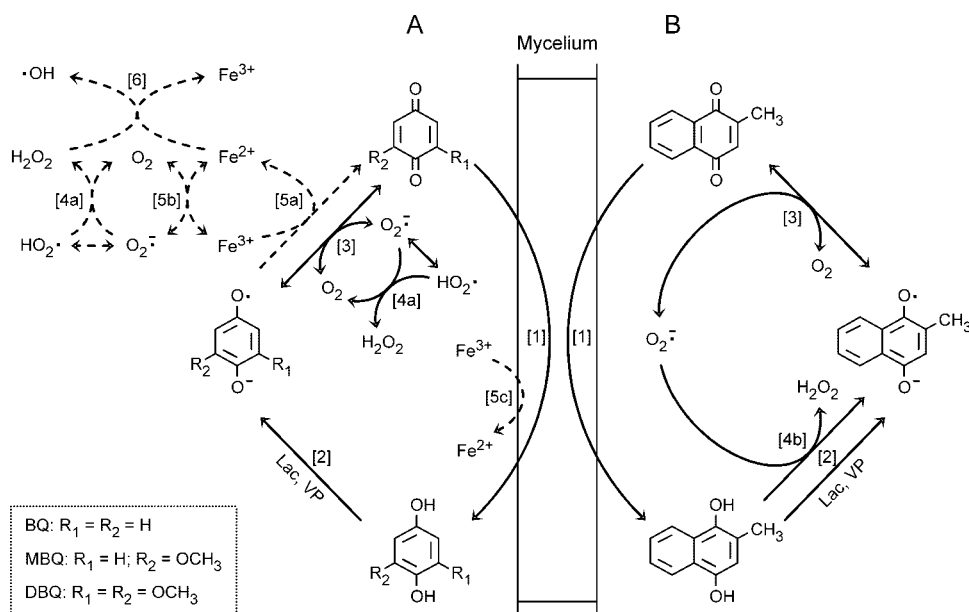


FIG. 8. Scheme of the quinone redox cycling process in *P. eryngii* (see Discussion for an explanation). (A) Main reactions involved in ROS production through BQ, MBQ, and DBQ redox cycling in the absence and presence of Fe³⁺-EDTA (solid and dashed arrows, respectively). (B) MD redox cycling, showing hydroquinone propagation by O₂^{•-}. Reversible reactions are indicated by double arrows.

ment due to the two-electron reduction of Q and the secretion of the resulting QH₂.

Quinone redox cycling in white-rot fungi is triggered by the actions of cell-bound enzymatic systems that reduce them to QH₂ (Fig. 8, reaction 1). Studies carried out mainly with *P. chrysosporium* have described the existence of two different systems reducing quinones, i.e., intracellular dehydrogenases using NAD(P)H as electron donors [Q + NAD(P)H + H⁺ → QH₂ + NAD(P)⁺] (6, 10) and a plasma membrane redox system (43). Two similar intracellular quinone reductases have been characterized in the brown-rot fungus *Gloeophyllum trabeum* (9). Our current research on the contributions of these systems to the reduction of quinones by *P. eryngii* has revealed that the plasma membrane redox system is not involved (data not shown). Following Q reduction, extracellular QH₂ oxidation presents several possibilities. Being phenolic compounds, QH₂s are susceptible to oxidation by any of the ligninolytic enzymes (Fig. 8, reaction 2). As inferred from the results shown in Fig. 1, 6, and 7, it is clear that oxidation of QH₂ by laccase (4 QH₂ + O₂ → 4 Q^{•-} + 2 H₂O + 4 H⁺) and peroxidases (2 QH₂ + H₂O₂ → 2 Q^{•-} + H₂O) increases the rates of quinone redox cycles and ROS production, provided Q reduction is the limiting reaction of the cycle. Chemical transformation of QH₂ into Q^{•-} is also a possibility to consider, since other oxidants than enzymes are either present or produced in the course of redox cycling. First, QH₂ autoxidation (QH₂ + O₂ → Q^{•-} + O₂^{•-} + 2 H⁺), which is a spin-restricted reaction, has been documented to be catalyzed by transition metal ions (49). For instance, Fe³⁺, chelated or not with oxalate, has been shown to be the catalyst of QH₂ oxidation in the redox cycling process described in brown-rot fungi (24, 44, 48). In the present study, special emphasis was placed on the involvement of the ligninolytic enzymes in quinone redox cycling. This is the reason why an iron complex preventing QH₂ ox-

idation, such as Fe³⁺-EDTA (14), was used. The effect of replacing EDTA by oxalate on [•]OH production by *P. eryngii* via DBQ redox cycling has already been determined and will be described in a separate publication from this study. Second, conversion of QH₂ into Q^{•-} can be accomplished by comproportionation reaction (QH₂ + Q ⇌ 2 Q^{•-} + 2 H⁺). This reaction has been demonstrated to trigger the “autoxidation” of several benzo- and naphthoquinones and is especially noticeable with QH₂ substituted with strong electron-donating groups, including DBQH₂ (37). The contribution of this reaction to the production of ROS could be evidenced in the absence of other enzymatic or chemical QH₂ oxidants. For instance, the production of [•]OH by *P. chrysosporium* during the DBQ redox cycle without producing any ligninolytic enzyme (Fig. 7) could be explained on the basis of this comproportionation reaction taking place. Third, the O₂^{•-} derived from Q^{•-} autoxidation can propagate the oxidation of some QH₂: QH₂ + O₂^{•-} → Q^{•-} + H₂O₂ (Fig. 8, reaction 4b). The existence of this reaction with the QH₂ derived from the reduction of the four Qs studied here was previously determined by studying the effect of superoxide dismutase on either the rate of QH₂ “autoxidation” or oxidation by laccase (16, 17). A negative effect indicative of suppression of QH₂ propagation by O₂^{•-} was observed only in the case of MDH₂, in agreement with other reported studies (35).

In the absence of Fe³⁺-EDTA, the pronounced increase exerted by quinones on the production of extracellular H₂O₂ by *P. eryngii* (Fig. 2) evidenced the transformation of Q^{•-} into Q via autoxidation (Q^{•-} + O₂ ⇌ Q + O₂^{•-} [Fig. 8, reaction 3]), followed by O₂^{•-} dismutation (O₂^{•-} + HO₂[•] + H⁺ → O₂ + H₂O₂ [Fig. 8, reaction 4a]) and, in the case of MD, also by O₂^{•-} reduction by MDH₂ (reaction 4b). As Q^{•-} autoxidation is a reversible reaction, the continuous removal of both quinones by fungal reducing systems (Fig. 1) and of O₂^{•-} by reactions 4a

and b were among the factors contributing to the efficiency of quinone redox cycling as an H_2O_2 production mechanism. This was previously demonstrated in laccase reactions with MBQH₂ and DBQH₂ by the recycling of MBQ and DBQ with diaphorase (a reductase catalyzing the divalent reduction of quinones) (17). In this way, transformation of Q^{•-} into Q via autoxidation was favored over two competing reactions (not shown in Fig. 8): Q^{•-} dismutation ($2 \text{Q}^{\bullet-} + 2 \text{H}^+ \rightleftharpoons \text{QH}_2 + \text{Q}$) and Q^{•-} laccase oxidation ($4 \text{Q}^{\bullet-} + \text{O}_2 + 4 \text{H}^+ \rightarrow 4 \text{Q} + 2 \text{H}_2\text{O}$). With respect to the removal of O₂^{•-} via spontaneous dismutation under the incubation conditions used here in redox cycling experiments (pH 5), it is quite likely that this was occurring around its optimum pH, as it implies the oxidation of O₂^{•-} by the hydroperoxyl radical (HO₂[•]) in equilibrium with O₂^{•-}, and the pK value of HO₂[•] is 4.8 (5). In the presence of Fe³⁺-EDTA, the high enhancement of the rates of Fe³⁺ reduction (Fig. 3), TBARS production (Fig. 4), and 4-hydroxybenzoic acid hydroxylation (Fig. 5) caused by Q evidenced the production of [•]OH by *P. eryngii* via Fenton's reagent formation. Reactions involved in this process are shown in Fig. 8. Under these conditions, Q^{•-} autoxidation is mainly catalyzed by Fe³⁺ (sum of reactions 5a, $\text{Q}^{\bullet-} + \text{Fe}^{3+} \rightarrow \text{Q} + \text{Fe}^{2+}$, and 5b, $\text{Fe}^{2+} + \text{O}_2 \rightleftharpoons \text{Fe}^{3+} + \text{O}_2^{\bullet-}$), leading to the production of [•]OH radicals by a Q^{•-}-driven Fenton reaction (reactions 5a and 6) (14). Two other pathways that must be considered to contribute to [•]OH generation, but to a lesser extent, are the O₂^{•-}-driven Fenton reaction (reactions reverse 5b and 6), using the O₂^{•-} produced by direct Q^{•-} autoxidation (reaction 3), and that caused by the reduction of Fe³⁺ by the unknown cell-bound system whose existence was inferred above from the results shown in Fig. 3 to 5 with no Q (reactions 5c and 6). Possible cell-bound systems catalyzing Fe³⁺ reduction are the plasma membrane redox system described in *P. chrysosporium* (42) and the plasma membrane reductase (Fre1 protein) involved in iron uptake by several fungi (26). In this regard, a possible role in controlling iron levels available for Fenton chemistry has been suggested for two *P. chrysosporium* ferroxidase activities, one of which is extracellular (28) and the other a component of the cell-bound iron uptake complex Fet3-Ftr1 (27).

One interesting characteristic of quinone redox cycling is the low substrate specificity of the enzymes participating in the process. The results obtained in the present study with three of the most representative quinones produced during the degradation of both softwood and hardwood lignins enhances the likelihood of extracellular ROS production by white-rot fungi through this mechanism. Quinones have been also shown to be common intermediates during the degradation of aromatic pollutants by these fungi (36). In this regard, it is likely that some of these quinone intermediates can act as redox cycling agents contributing to ROS production and thus to the degradation of the pollutant from which they derive. This could be the case with MD being an oxidation product of 2-methylnaphthalene. The high correlation observed between the levels of H₂O₂ (Fig. 2) and [•]OH radicals (Fig. 4 and 5) allows us to use the same rationales to explain the differences found in both cases with the different quinones. It is quite likely that O₂^{•-} reduction by MDH₂ (Fig. 8, reaction 4b) was the main factor contributing to the production of the highest levels of H₂O₂ and [•]OH during MD redox cycling for two reasons. First, it doubles the amount of H₂O₂ produced by O₂^{•-} dismutation,

which is the only reaction converting O₂^{•-} into H₂O₂ with the three benzoquinones. Second, it increases the rate of QH₂ oxidation, which is the limiting reaction of MBQ and DBQ redox cycles, as inferred from the results shown in Fig. 1, and the BQ redox cycle, as previously reported by Guillén et al. (16). In fact, the latter study showed that the concerted action of laccase and O₂^{•-} increased MDH₂ oxidation in such a way that MD reduction became the limiting reaction of its redox cycle. The differences observed in the extents of H₂O₂ and [•]OH production during the redox cycles of the three benzoquinones (DBQ > MBQ > BQ) can be explained by considering the ability of Q^{•-} radicals to be autoxidized. This ability, estimated previously as the production of H₂O₂ during the oxidation of DBQH₂, MBQH₂ and BQH₂ by purified laccase of *P. eryngii*, was shown to be DBQ^{•-} > MBQ^{•-} > BQ^{•-} (16, 17). Furthermore, the higher the laccase and VP activities on QH₂, which have been reported to be DBQH₂ > MBQH₂ > BQH₂ (13, 17), the higher the rate of the quinone redox cycle.

The induction of TBARS production by DBQ and Fe³⁺-EDTA in *B. adusta*, *P. chrysosporium*, *P. radiata*, *P. cinnabarinus*, and *T. versicolor* (Fig. 7) led us to conclude that quinone redox cycling is a widespread mechanism for ROS production among white-rot fungi, including O₂^{•-}, H₂O₂, and [•]OH radicals. This process can be operative in the absence of ligninolytic enzymes provided other QH₂ oxidants are present, as reported with brown-rot fungi (24, 48) and shown here in the case of *P. chrysosporium*. However, the significance of QH₂ enzymatic catalysis has been clearly evidenced by the following observations: the increase exerted by added laccase and VP on TBARS production by *P. eryngii* (Fig. 6) and the much higher TBARS production rates obtained with fungi expressing ligninolytic enzymes under the culture conditions used (Table 2 and Fig. 7). From the results shown in Fig. 4 and 7, it can be inferred that the enzyme playing a crucial role in terms of [•]OH production was laccase. First, TBARS production by *P. eryngii* was found to be higher with Lac-mycelium than with LacVP-mycelium (Fig. 4). Second, TBARS production with the fungi expressing only laccase (*P. radiata*, *P. cinnabarinus*, and *T. versicolor*) was found to be higher than with the fungus expressing only peroxidases (*B. adusta*) (Fig. 7). This is not surprising, because oxidation of QH₂ by peroxidases implies the consumption of part of the H₂O₂ required for [•]OH generation. The participation of laccase in the production of highly reactive oxidants deserves more attention, since it could explain the solubilization and mineralization of lignin observed with some ascomycetes producing only this ligninolytic enzyme (29, 40).

In summary, the results shown in the present study provide new information on the mechanisms used by white-rot fungi to activate O₂ in the extracellular environment. In addition, they supply the basis for a simple strategy that could be used in fundamental and practical studies, such as the determination of factors affecting [•]OH production by quinone redox cycling, as well as the roles these radicals can play in lignin and organopollutant degradation by these fungi. In this regard, our current research in this field, carried out with *P. eryngii* and *T. versicolor*, is showing that the induction of extracellular [•]OH radical production by quinones and iron occurs not only in incubations with washed mycelium, but also with whole fungal cultures during primary and secondary metabolism. This re-

search is also revealing that the capability of these fungi to degrade pollutants is increased when quinone redox cycling is used as a strategy to induce the production of extracellular $\cdot\text{OH}$ radicals (unpublished data).

ACKNOWLEDGMENTS

The research was funded by two projects from the Comunidad de Madrid (GR/AMB/0812/2004 and S-0505/AMB0100) and one project from the Comunidad de Madrid-Universidad de Alcalá (CAM-UAH2005/065). The stays of V. Gómez-Toribio at the Centro de Investigaciones Biológicas and A. B. García-Martín at the Universidad de Alcalá were supported, respectively, by fellowships from the Comunidad de Madrid and the Universidad de Alcalá.

We thank P. R. Shearer for English revision.

REFERENCES

- Backa, S., J. Gierer, T. Reitberger, and T. Nilsson. 1993. Hydroxyl radical activity associated with the growth of white-rot fungi. *Holzforschung* **47**:181–187.
- Baker, W. 1941. Derivatives of pentahydroxybenzene, and a synthesis of pedicellin. *J. Chem. Soc.* **1941**:662–670.
- Baldrian, P., and V. Valaskova. 2008. Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiol. Rev.* **32**:501–521.
- Barr, D. P., M. M. Shah, T. A. Grover, and S. D. Aust. 1992. Production of hydroxyl radical by lignin peroxidase from *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* **298**:480–485.
- Bielski, B. H. J., and A. O. Allen. 1977. Mechanism of the disproportionation of superoxide radicals. *J. Phys. Chem.* **81**:1048–1050.
- Brock, B. J., S. Rieble, and M. H. Gold. 1995. Purification and characterization of a 1,4-benzoquinone reductase from the basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **61**:3076–3081.
- Camarero, S., D. Ibarra, M. J. Martínez, and A. T. Martínez. 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl. Environ. Microbiol.* **71**:1775–1784.
- Cohen, R., K. A. Jensen, C. J. Houtman, and K. E. Hammel. 2002. Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. *FEBS Lett.* **531**:483–488.
- Cohen, R., M. R. Suzuki, and K. E. Hammel. 2004. Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* **70**:324–331.
- Constam, D., A. Muheim, W. Zimmermann, and A. Fiechter. 1991. Purification and partial characterization of an intracellular NADH:quinone oxidoreductase from *Phanerochaete chrysosporium*. *J. Gen. Microbiol.* **137**:2209–2214.
- Cullen, D., and P. J. Kersten. 2004. Enzymology and molecular biology of lignin degradation, p. 249–312. In R. Braml and G. A. Marzluf (ed.), *The Mycota. III. Biochemistry and molecular biology*. Springer-Verlag, Berlin, Germany.
- Forney, L. J., C. A. Reddy, M. Tien, and S. D. Aust. 1982. The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. *J. Biol. Chem.* **257**:11455–11462.
- Gómez-Toribio, V., A. T. Martínez, M. J. Martínez, and F. Guillén. 2001. Oxidation of hydroquinones by the versatile ligninolytic peroxidase from *Pleurotus eryngii*: H_2O_2 generation and the influence of Mn^{2+} . *Eur. J. Biochem.* **268**:4787–4793.
- Guillén, F., V. Gómez-Toribio, M. J. Martínez, and A. T. Martínez. 2000. Production of hydroxyl radical by the synergistic action of fungal laccase and aryl alcohol oxidase. *Arch. Biochem. Biophys.* **383**:142–147.
- Guillén, F., M. J. Martínez, and A. T. Martínez. 1996. Hydroxyl radical production by *Pleurotus eryngii* via quinone redox-cycling, p. 389–392. In K. Messner and E. Srebotnik (ed.), *Biotechnology in the pulp and paper industry: recent advances in applied and fundamental research*. Facultas-Universitätsverlag, Vienna, Austria.
- Guillén, F., M. J. Martínez, C. Muñoz, and A. T. Martínez. 1997. Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. *Arch. Biochem. Biophys.* **339**:190–199.
- Guillén, F., C. Muñoz, V. Gómez-Toribio, A. T. Martínez, and M. J. Martínez. 2000. Oxygen activation during the oxidation of methoxyhydroquinones by laccase from *Pleurotus eryngii*. *Appl. Environ. Microbiol.* **66**:170–175.
- Gutteridge, J. M. C. 1984. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochem. J.* **224**:761–767.
- Haemmerli, S. D., H. E. Schoemaker, H. W. H. Schmidt, and M. S. A. Leisola. 1987. Oxidation of veratryl alcohol by the lignin peroxidase of *Phanerochaete chrysosporium*. Involvement of activated oxygen. *FEBS Lett.* **220**:149–154.
- Halliwell, B., and J. M. C. Gutteridge. 1981. Formation of a thiobarbituric acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Lett.* **128**:347–352.
- Hammel, K. E., A. N. Kapich, K. A. Jensen, and Z. C. Ryan. 2002. Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb. Technol.* **30**:445–453.
- Johannes, C., and A. Majcherczyk. 2000. Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. *Appl. Environ. Microbiol.* **66**:524–528.
- Kappus, H., and H. Sies. 1981. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**:1233–1241.
- Kerem, Z., K. A. Jensen, and K. E. Hammel. 1999. Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven Fenton reaction. *FEBS Lett.* **446**:49–54.
- Kimura, Y., Y. Asada, and M. Kuwahara. 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. *Appl. Microbiol. Biotechnol.* **32**:436–442.
- Kosman, D. J. 2003. Molecular mechanisms of iron uptake in fungi. *Mol. Microbiol.* **47**:1185–1197.
- Larrondo, L. F., P. Canessa, F. Melo, R. Polanco, and R. Vicuna. 2007. Cloning and characterization of the genes encoding the high-affinity iron-uptake protein complex Fet3/Ftr1 in the basidiomycete *Phanerochaete chrysosporium*. *Microbiology* **153**:1772–1780.
- Larrondo, L. F., L. Salas, F. Melo, R. Vicuña, and D. Cullen. 2003. A novel extracellular multicopper oxidase from *Phanerochaete chrysosporium* with ferroxidase activity. *Appl. Environ. Microbiol.* **69**:6257–6263.
- Liers, C., R. Ulbrich, K. T. Steffen, A. Hatakka, and M. Hofrichter. 2006. Mineralization of ^{14}C -labelled synthetic lignin and extracellular enzyme activities of the wood-colonizing ascomycetes *Xylaria hypoxylon* and *Xylaria polymorpha*. *Appl. Microbiol. Biotechnol.* **69**:573–579.
- Martínez, A. T. 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme Microb. Technol.* **30**:425–444.
- Martínez, A. T., M. Speranza, F. J. Ruiz-Dueñas, P. Ferreira, S. Camarero, F. Guillén, M. J. Martínez, A. Gutiérrez, and J. C. del Río. 2005. Biodegradation of lignocelluloses: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* **8**:195–204.
- Martínez, M. J., F. J. Ruiz-Dueñas, F. Guillén, and A. T. Martínez. 1996. Purification and catalytic properties of two manganese-peroxidase isoenzymes from *Pleurotus eryngii*. *Eur. J. Biochem.* **237**:424–432.
- Mason, M. G., P. Nicholls, and M. T. Wilson. 2003. Rotting by radicals—the role of cellobiose oxidoreductase? *Biochem. Soc. Trans.* **31**:1335–1336.
- Muñoz, C., F. Guillén, A. T. Martínez, and M. J. Martínez. 1997. Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl. Environ. Microbiol.* **63**:2166–2174.
- Öllinger, K., G. D. Buffinton, L. Ernster, and E. Cadenas. 1990. Effect of superoxide dismutase on the autoxidation of substituted hydro- and seminaaphthoquinones. *Chem. Biol. Interact.* **73**:53–76.
- Pointing, S. B. 2001. Feasibility of bioremediation by white-rot fungi. *Appl. Microbiol. Biotechnol.* **57**:20–33.
- Roginsky, V. A., L. M. Pisarenko, W. Bors, and C. Michel. 1999. The kinetics and thermodynamics of quinone-semiquinone-hydroquinone systems under physiological conditions. *J. Chem. Soc. Perkin Trans.* **2**:871–876.
- Ruiz-Dueñas, F. J., F. Guillén, S. Camarero, M. Pérez-Boada, M. J. Martínez, and A. T. Martínez. 1999. Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus *Pleurotus eryngii*. *Appl. Environ. Microbiol.* **65**:4458–4463.
- Saparrat, M. C. N., F. Guillén, A. M. Arambarri, A. T. Martínez, and M. J. Martínez. 2002. Induction, isolation, and characterization of two laccases from the white-rot basidiomycete *Coriolopsis rigida*. *Appl. Environ. Microbiol.* **68**:1534–1540.
- Shary, S., S. A. Ralph, and K. E. Hammel. 2007. New insights into the ligninolytic capability of a wood decay ascomycete. *Appl. Environ. Microbiol.* **73**:6691–6694.
- Somogyi, M. 1945. A new reagent for the determination of sugars. *J. Biol. Chem.* **160**:61–73.
- Stahl, J. D., and S. D. Aust. 1995. Properties of a transplasma membrane redox system of *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* **320**:369–374.
- Stahl, J. D., S. J. Rasmussen, and S. D. Aust. 1995. Reduction of quinones and radicals by a plasma membrane redox system of *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* **322**:221–227.
- Suzuki, M. R., C. G. Hunt, C. J. Houtman, Z. D. Dalebroux, and K. E. Hammel. 2006. Fungal hydroquinones contribute to brown rot of wood. *Environ. Microbiol.* **8**:2214–2223.
- Tanaka, H., S. Itakura, and A. Enoki. 1999. Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Holzforschung* **53**:21–28.
- Tien, M., and T. K. Kirk. 1984. Lignin-degrading enzyme from *Phanero-*

- chaete chrysosporium*: purification, characterization, and catalytic properties of a unique H₂O₂-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81**:2280–2284.
47. **Tortella, G. R., M. C. Diez, and N. Durán.** 2005. Fungal diversity and use in decomposition of environmental pollutants. Crit. Rev. Microbiol. **31**:197–212.
48. **Varela, E., and M. Tien.** 2003. Effect of pH and oxalate on hydroquinone-derived hydroxyl radical formation during brown rot wood degradation. Appl. Environ. Microbiol. **69**:6025–6031.
49. **Zhang, L. P., B. Bandy, and A. J. Davison.** 1996. Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol. Free Radic. Biol. Med. **20**:495–505.