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

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A simple strategy for the induction of extracellular hydroxyl radical (·OH) production by white-rot fungi is presented. It involves the incubation of mycelium with quinones and Fe3+-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 ·OH production by a Fenton reaction (H2O2 + Fe3+ → ·OH + OH− + Fe2+). H2O2 production, Fe3+ reduction, and ·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-benzohydroquinone (MBQ), 2,6-dimethoxy-1,4-benzoquinone (DBQ), and 2-methyl-1,4-naphthoquinone (menadione [MD]). In all cases, ·OH radicals were linearly produced, with the highest rate obtained with MD, followed by DBQ, MBQ, and BQ. These rates correlated with both H2O2 levels and Fe3+ 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 ·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 ·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 ·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) Mn3+, (ii) free radicals from some fungal metabolites and lignin depolymerization products (7, 22), and (iii) oxygen free radicals, mainly hydroxyl radicals (·OH) and lipid peroxidation radicals (21). Although ·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 ·OH production still await in vivo validation.

Several potential sources of extracellular ·OH based on the Fenton reaction (H2O2 + Fe2+ → ·OH + OH− + Fe3+) have been postulated for white-rot fungi. In one case, an extracellular fungal glycopeptide has been shown to reduce O2 and Fe3+ to H2O2 and Fe2+ (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 ·OH through the generation of Fe3+ and O2 reductants, such as formate (CO2−2) and semiquinone (Q−) radicals. The first time evidence was provided that a ligninolytic enzyme was involved in ·OH production, oxalate was used to generate CO2−2 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 (Valc+) by LiP, oxidation of oxalate to CO2−2 by Valc+, reduction of O2 to O2− by CO2−2, and a superoxide-driven Fenton reaction (Haber-Weiss reaction) in which Fe3+ was reduced by O2−. The ·OH production mechanism assisted by Q− was inferred from the oxidation of 2-methoxy-1,4-benzohydroquinone (MBOH2) and 2,6-dimethoxy-1,4-benzoquinone (DBQH2) by Pleurotus eryngii laccase in the presence of Fe3+-EDTA. The ability of Q− radicals to reduce both Fe3+ to Fe2+ and O2 to O2−, which dismutated to H2O2, was demonstrated (14). In this case, ·OH radicals were generated by a semiquinone-driven Fenton reaction, as Q− radicals were the main agents accomplishing Fe3+ reduction. The first evidence of the likelihood of this ·OH production mechanism being operative in vivo had been obtained from incubations of P. eryngii with 2-methyl-1,4-naphthoquinone (menadione [MD]) and Fe3+-EDTA (15). Extracellular ·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...
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-benzoquinone (BQH₂), and MBQH₂ were from Aldrich. DBOH₂ was prepared from DBQ by reduction with sodium borohydride (2). DBQH₂ 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 IJFIM A169 (Fungal Culture Collection of the Centro de Investigaciones Biológicas) (≈ ATCC 90787 and CBS 613.91), Trametes versicolor IJFIM A136, Phlebia radiata CBS AS88 (≈ CBS 184.83), Bkerkandea adusta IJFIM AS81 (≈ CBS 595.78), and Pycnoporus cinnabarinus IJFIM AT670 (≈ 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 shaker (150 rpm) 250-ml conical flasks with 100 ml of a glucose-peptone mixture cooled, the absorbance was read at 532 nm against appropriate blanks. Mycelial pellets were produced at 28°C in shaker (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 (GPMs medium). Incocula 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 coevuligone (extraction coefficient at 469 nm ε₃₁₀ = 27,500 M⁻¹ cm⁻¹, when referring to the DMP concentration) (32). VP and MnP activities were estimated by Mn⁴⁺-tartrate complex formation (ε₅₄₆ = 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 (ε₃₉₀ = 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, BOH₂ was selected, since laccase activity on BOH₂ has been shown to be quite low (34). QR activity was determined in 50 mM phosphate buffer, pH 5, using 500 μM BOH₂ as a substrate and measuring the production of BOH₂ by high-performance liquid chromatography (HPLC). Samples (20 μl) were injected into a Pharmacia system equipped with a Spherisorb S5DS2 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 BOH₂ 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 shaker 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 BOH₂, 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 FeCl₃-110 mM 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, protocatechuate 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 S5DS2 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 and 0.1 M 0.1% standard commercial NaOH solution, which were calculated spectrophotometrically (ε₅₃₂ = 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 protocatechuate 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) thioracilonic 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). Protocatechuate 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 chelate 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

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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 Mn$^{2+}$ in the culture medium 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 QH$_2$s was observed during the first 20 min. Then, QH$_2$/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$^{-1}$, QH$_2$/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 DBQH$_2$ than MBQH$_2$ (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 H$_2$O$_2$ and reduction of Fe$^{3+}$ by P. eryngii upon incubation with BQ, MBQ, DBQ, and MD was evaluated as a requisite for $\cdot$OH generation. Figure 2A and B shows the results for H$_2$O$_2$ production by LacVP-mycelium and Lac-mycelium, respectively. Regardless of the mycelium used, basal

### 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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Enzyme</th>
<th>Activity</th>
<th>Culture liquid (mU ml$^{-1}$)</th>
<th>Cell bound (mU mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td>Lac</td>
<td>24 ± 2</td>
<td>8 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP</td>
<td>330 ± 13</td>
<td>4 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QR</td>
<td>ND$^a$</td>
<td>30 ± 3</td>
<td></td>
</tr>
<tr>
<td>GPMn</td>
<td>Lac</td>
<td>57 ± 5</td>
<td>11 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QR</td>
<td>ND$^a$</td>
<td>28 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ ND, not determined.

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, MBQH$_2$ and DBQH$_2$). 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 H$_2$O$_2$ 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 H$_2$O$_2$ were also carried out in the absence of quinones. The error bars indicate standard deviations.
levels of extracellular H$_2$O$_2$ 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$_2$O$_2$ levels until steady values were attained (after 30 min). The average levels of 60- to 180-min samples increased in the order BQ > MBQ > DBQ > 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$_2$O$_2$, it nearly disappeared from the extracellular liquid in about 60 min. After this time, H$_2$O$_2$ levels were similar to those found without quinones (2 to 3 μM). Although this decrease in H$_2$O$_2$ 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$_2$O$_2$ 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 calculated from 0- to 60-min samples were 0.1 and 0.4 μM 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 μM min$^{-1}$ (LacVP-mycelium) and 0.9 μM 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 μM min$^{-1}$ with LacVP-mycelium and 5.0 μM 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 OH$^-$ was studied in incubations of washed mycelia with quinones and Fe$^{3+}$-EDTA. Depending on the procedure used to detect 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. 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.](http://aem.asm.org)

![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.](http://aem.asm.org)
TBARS levels and those of H₂O₂ shown in Fig. 2. Furthermore, taking into account that Fe²⁺ was produced at a rate similar to those of MBQ, DBQ, and MD (Fig. 3), it could be inferred that H₂O₂ 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 Fe³⁺-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 Fe³⁺ with no Q. In addition to H₂O₂ basal levels generated by the fungus under these conditions (Fig. 2), 'OH production could be supported by that derived from ferrous iron autoxidation, followed by the dismutation of the resulting O₂⁻⁻⁻.

In order to confirm 'OH production, the effect of 'OH scavengers, such as mannitol and dimethyl sulfoxide (DMSO), on TBARS formation was evaluated. Lac-mycelium was incubated with DBQ, Fe³⁺-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 μM min⁻¹ (incubation without scavengers) to 7.4 and 2.8 μM min⁻¹ with mannitol and DMSO, respectively. The production of '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 during the incubation of Lac-mycelium with DBQ, Fe³⁺-EDTA, and 2-deoxyribose, and samples were analyzed for TBARS 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 Fe³⁺–110 μM EDTA, and 2.8 mM 2-deoxyribose, in the absence (0 μU) and presence of 50 and 100 μU ml⁻¹ laccase (solid lines) and VP (dashed lines). The error bars indicate standard deviations.

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Laccase (μU ml⁻¹)</th>
<th>MnP/VP (μU ml⁻¹)</th>
<th>LiP (μU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. adusta</td>
<td>0</td>
<td>38 ± 3</td>
<td>192 ± 15</td>
</tr>
<tr>
<td>P. chrysosporium</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. radiata</td>
<td>319 ± 22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. cinnabarinus</td>
<td>19 ± 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T. versicolor</td>
<td>217 ± 14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
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 *Coriolopsis 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 O\(_2\)\(^{-}\) and H\(_2\)O\(_2\) 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 Fe\(^{2+}\) and H\(_2\)O\(_2\) (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 napthoquinone (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 MDH\(_2\) oxidation by O\(_2\)\(^{-}\) (reaction 4b), which, as discussed below, was probably the factor contributing most to the production of the highest levels of H\(_2\)O\(_2\) and 'OH radicals with this quinone (Fig. 2 and 4, respectively). With the exception of the enzymatic oxidation of QH\(_2\), a similar quinone redox cycling process has been described in brown-rot fungi. These fungi produce several methoxyhydroquinones that can be chemically oxidized by Fe\(^{3+}\) (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 QH\(_2\) 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. 7. Hydroxyl radical production by selected white-rot fungi via DBQ redox cycling. Levels of DBQ (A), DBQH\(_2\) (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 Fe\(^{3+}\)–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 A\(_{532}\) 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 DBQH\(_2\)/DBQ ratios found at system equilibrium (Fig. 7A and B), similar to the results obtained with *P. eryngii* (Fig. 1). Thus, whereas DBQH\(_2\) was the prevalent compound 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 A\(_{532}\) min\(^{-1}\)), producing only laccinolytic peroxidases. However, it was four times higher than that observed with *P. chrysosporium*, indicating the involvement of these enzymes in DBQ redox cycling.
ment due to the two-electron reduction of Q and the secretion of the resulting OH2.

Quinone redox cycling in white-rot fungi is triggered by the actions of cell-bound enzymatic systems that reduce them to OH2 (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+ → OH2 + 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 OH2 oxidation presents several possibilities. Being phenolic compounds, OH2s 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 OH2 by laccase (4 OH2 + O2 → 4 O− + 2 H2O + 4 H+) and peroxidases (2 OH2 + H2O2 → 2 O− + H2O) increases the rates of quinone redox cycles and ROS production, provided Q reduction is the limiting reaction of the cycle. Chemical transformation of OH2 into O− is also a possibility to consider, since other oxidants than enzymes are either present or produced in the course of redox cycling. First, OH2 autoxidation (OH2 + O2 → O− + 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 OH2 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 OH2 oxidation, 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 OH2 into O− has already been accomplished and will be described in a separate publication from this study. Second, conversion of OH2 into O− has already been accomplished and will be described in a separate publication from this study. Second, conversion of OH2 into O− has already been accomplished and will be described in a separate publication from this study. Second, conversion of OH2 into O− has already been accomplished and will be described in a separate publication from this study. Second, conversion of OH2 into O− has already been accomplished and will be described in a separate publication from this study. 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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 O− 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 O− autoxidation is a reversible reaction, the continuous removal of both quinones by fungal reducing systems (Fig. 1) and of O2− by reactions 4a

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, MBO, 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.
and b were among the factors contributing to the efficiency of quinone redox cycling as an H₂O₂ 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 divergent 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 Q− + 2 H⁺ ⇌ OH₂ + O) and Q− laccase oxidation (4 Q− + O₂ + 4 H⁺ → 4 O + 2 H₂O). With respect to the removal of O₂− via spontaneous dismutase 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 pH 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, Q− + Fe³⁺ → Q + Fe²⁺), and 5b, Fe²⁺ + O₂ ⇌ Fe³⁺ + O₂−), 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-methylphenanthrene. The high correlation observed between the levels of H₂O₂ (Fig. 2) and OH radicals (Fig. 4 and 5) allows us to use the same rationale 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 OH₂ oxidation, which is the limiting reaction of MBQ and DBQ redox cycles, as inferred from the results shown in Fig. 1, and the BO 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 > BO) 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 DBOH₂, MBQH₂ and BOH₂ by purified laccase of P. eryngii, was shown to be DBO− > MBQ− > BO− (16, 17). Furthermore, the higher the laccase and VP activities on OH₂, which have been reported to be DBOH₂ > MBQH₂ > BOH₂ (13, 17), the higher the rate of the quinone redox cycle.

The induction of TBARS production by DBQ and Fe²⁺-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 OH₂ oxidants are present, as reported with brown-rot fungi (24, 48) and shown here in the case of P. chrysosporium. However, the significance of OH₂ 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 OH₂ 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 ‘OH radicals (unpublished data).

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