Environ. Sci. Technol. **2007**, *41,* 2964−2971

Transformation of Polycyclic Aromatic Hydrocarbons by Laccase Is Strongly Enhanced by Phenolic Compounds Present in Soil

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Efficient transformation of several polycyclic aromatic hydrocarbons (PAHs) was obtained using a fungal laccase in the presence of phenolic compounds related to those formed in nature during the turnover of lignin and humus. The effect of these natural mediators, namely vanillin, acetovanillone, acetosyringone, syringaldehyde, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, and sinapic acid, was compared with that of synthetic mediators such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT). Anthracene was significantly degraded by laccase in the absence of mediators, whereas benzo[a]pyrene and pyrene were weakly transformed (less than 15% after 24 h). Vanillin, acetovanillone, 2,4,6-trimethylphenol, and, above all, *p*-coumaric acid strongly promoted the removal of PAHs by laccase. 9,10-Anthraquinone was the main product detected from anthracene oxidation by all the laccase−mediator systems. The yield of anthraquinone formed was directly correlated with the amount of *p*-coumaric acid used. This compound resulted in a better laccase mediator than ABTS and close similarity to HBT, attaining 95% removal of anthracene and benzo[a]pyrene and around 50% of pyrene within 24 h. Benzo[a]pyrene 1,6-, 3,6-, and 6,12-quinones were produced during benzo[a]pyrene oxidation with laccase and *p*-coumaric acid, HBT, or ABTS as mediators, although use of the latter mediator gave further oxidation products that were not produced by the two other systems.

Introduction

Secretion of extracellular oxidoreductases confers ligninolytic basidiomycetes a unique capability to degrade lignin polymer in wood as well as to detoxify a variety of recalcitrant aromatic compounds. Polycyclic aromatic hydrocarbons (PAHs) are widely spread pollutants which toxicity and recalcitrance to microbial degradation correlate with the number and angularity of their fused benzene rings (*1*, *2*). Unlike other fungi, white-rot basidiomycetes can mineralize PAHs (*3*). Although cytochrome P-450 type monooxygenases might also be involved in the oxidation of PAHs by these fungi (*1*), there is strong evidence about the involvement of ligninolytic peroxidases and laccases in PAHs oxidation to quinones, a key step in PAH degradation by white-rot fungi (*4*−*6*), and other transformation reactions including aromatic-ring opening (*7*, *8*). Quinones are less toxic than the respective PAH (*9*), by contrast to carcinogenic and mutagenic dihydroxylated metabolites generated by cytochrome P-450 monooxygenases (*10*). Therefore, enzymatic oxidation of PAH by ligninolytic oxidoreductases could be a useful strategy in detoxification and bioremediation processes.

Laccases are multicopper oxidases produced by most white-rot fungi that oxidize a wide range of aromatic compounds, having high activity on substituted phenols (*11*). The use of molecular oxygen as a final electron acceptor promotes the applicability of these enzymes compared to ligninolytic peroxidases. Laccase limitations, derived from their relative low redox potential (*12*), have been overcome by using redox mediators like ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) or 1-hydroxybenzotriazole (HBT), allowing the oxidation of non-phenolic compounds with high-redox potentials (*13*). Both mediators promote the oxidation of PAH by laccase (*6*, *14*, *15*). However, the use of these synthetic compounds implies high added costs and the possible generation of toxic oxidized species, bringing about the convenience of searching for alternative mediators.

Lignin biodegradation is an oxidative process wherein enzymatic and radical reactions take place (*16*). Free radicals of some fungal metabolites and lignin-derived products could act as natural redox mediators of ligninolytic oxidoreductases. The contribution of these compounds would be especially noticeable in those fungi producing laccase as the main or sole ligninolytic oxidoreductase. Recently, we described the efficiency of a series of naturally occurring substituted phenols as laccase mediators in dye decolorization (*17*). Some of these compounds (e.g., *p*-hydroxycinnamic acids) are present in herbaceous plants as extractives or forming lignin-carbohydrate bridges (*18*). Others (including phenolic aldehydes, ketones, and acids) originate during lignin degradation and are subsequently incorporated into the soil organic matter (humus). The capabilities of some of these natural compounds, namely vanillin, acetovanillone, acetosyringone, syringaldehyde, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, and sinapic acid, to promote PAHs transformation by *Pycnoporus cinnabarinus* laccase are studied here and compared with those of ABTS and HBT.

Materials and Methods

Chemicals. Anthracene, 9,10-anthraquinone, benzo[a]pyrene, pyrene, HBT, vanillin, acetovanillone (4-hydroxy-3-methoxyphenylethanone), syringaldehyde, acetosyringone (4-hydroxy-3,5-dimethoxyphenylethanone), 2,4,6-trimethylphenol, *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), and sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (97−99% purity) were purchased from Sigma-Aldrich. ABTS (98% purity) was purchased from Roche.

Laccase Production and Purification. Laccase was produced by Beldem (Belgium) from hyperproducer *P. cinnabarinus ss3* (*19*) and purified according to (*17*).

Laccase activity was determined by monitoring 5 mM ABTS oxidation to its cation radical (ϵ_{436} 29 300 mM^{-1} cm^{-1}) in 100 mM acetate buffer, pH 5 at room temperature. One

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10.1021/es062328j CCC: $37.00

Published on Web 03/21/2007

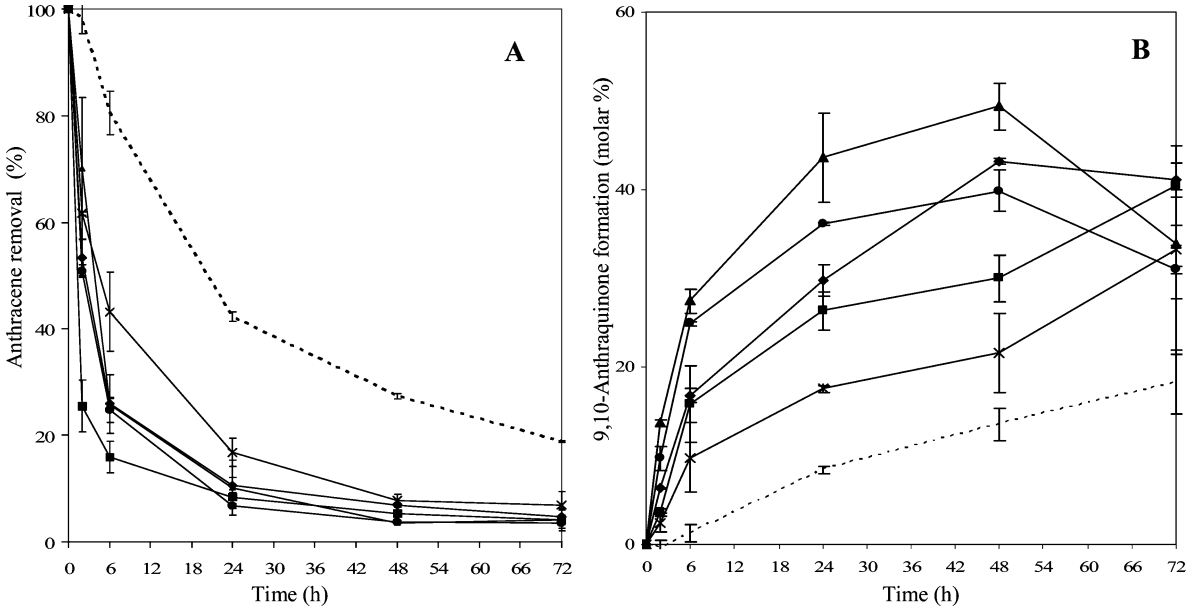

FIGURE 1. Effect of the concentration of *p*-coumaric acid on removal of 50 μM anthracene (A) and production of 9,10-anthraquinone (B) by *P. cinnabarinus* laccase. *p*-Coumaric acid /anthracene ratios of 1 (×), 2 (■), 4 (◆), 10 (●), and 20 (▲) and laccase without mediator (----) are shown (mean values and 95% confidence limits).

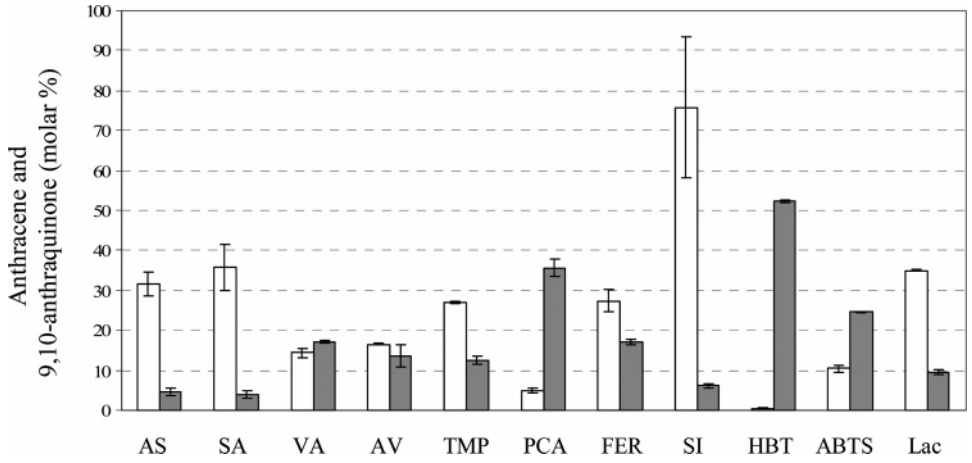

FIGURE 2. Molar percentages of residual anthracene (white bars) and 9,10-anthraquinone obtained (dark bars) after 24-h reaction of anthracene (50 μM) with laccase and different mediators (mediator/anthracene ratio of 10). Mean values and 95% confidence limits from HPLC areas are shown. Mediators: acetosyringone (AS), syringaldehyde (SA), vanillin (VA), acetovanillone (AV), 2,4,6-trimethylphenol (TMP), *p*-coumaric acid (PCA), ferulic acid (FER), sinapic acid (SI), HBT, and ABTS (Lac, laccase without mediator).

activity unit was defined as the amount of enzyme that releases 1 μmol of product per min.

Screening of Natural Laccase Mediators for Anthracene Oxidation. Anthracene (250 μM, in 2% acetone) oxidation by laccase (2 U/mL) in 100 mM sodium citrate buffer pH 5 (containing 1% Tween 20) was studied at room temperature, under continuous rolling (6 rev/min) and complete darkness. Acetosyringone, syringaldehyde, vanillin, acetovanillone, *p*-coumaric acid, and 2,4,6-trimethylphenol (500 μM) were tested as mediators and compared with HBT. Laccase without mediator was used as control. After 0, 4, 24, and 48 h, samples were collected for colorimetric determination of the 9,10-anthraquinone produced. Orange-colored 9,10-anthrahydroquinone from 9,10-anthraquinone reduction with sodium borohydride was measured at 419 nm (*20*).

Transformation of PAHs by Laccase in the Presence of Natural Mediators. Enzymatic reactions were carried out as described above using 50 μM anthracene, at 30 °C, under continuous rolling (6 rev/min) and complete darkness, in 2−4 mL Teflon-sealed vials. Samples were collected, and reactions were stopped with 50% acetonitrile and frozen. *p*-Coumaric acid (50, 100, 200, 500 μM, and 1 mM) was assayed to determine the effect of mediator concentration onto anthracene oxidation after 0, 2, 6, 24, 48, and 72 h of reaction.

Anthracene and benzo[a]pyrene transformation with laccase and acetosyringone, syringaldehyde, vanillin, acetovanillone, 2,4,6-trimethylphenol, *p*-coumaric acid (PCA), ferulic acid, sinapic acid, HBT, or ABTS (500 μM) as mediators, was carried out analogously as well as the comparative oxidation of anthracene, benzo[a]pyrene, and pyrene by laccase−PCA using 100 μM and 1 mM mediator. Reactions with boiled enzyme were used as negative controls in all the experiments. Removal of 10−15% of anthracene, 5−7% of pyrene, and 0−5% of benzo[a]pyrene after 24−48 h were-obtained, respectively. Data from enzymatic reactions after subtraction of abiotic controls are shown in the figures.

High Performance Liquid Chromatography (HPLC) Analyses. Separation of PAHs and their oxidation products were performed by reverse-phase HPLC. Transformation rates and production of 9,10-anthraquinone were calculated from peak areas using standards. Analyses were performed

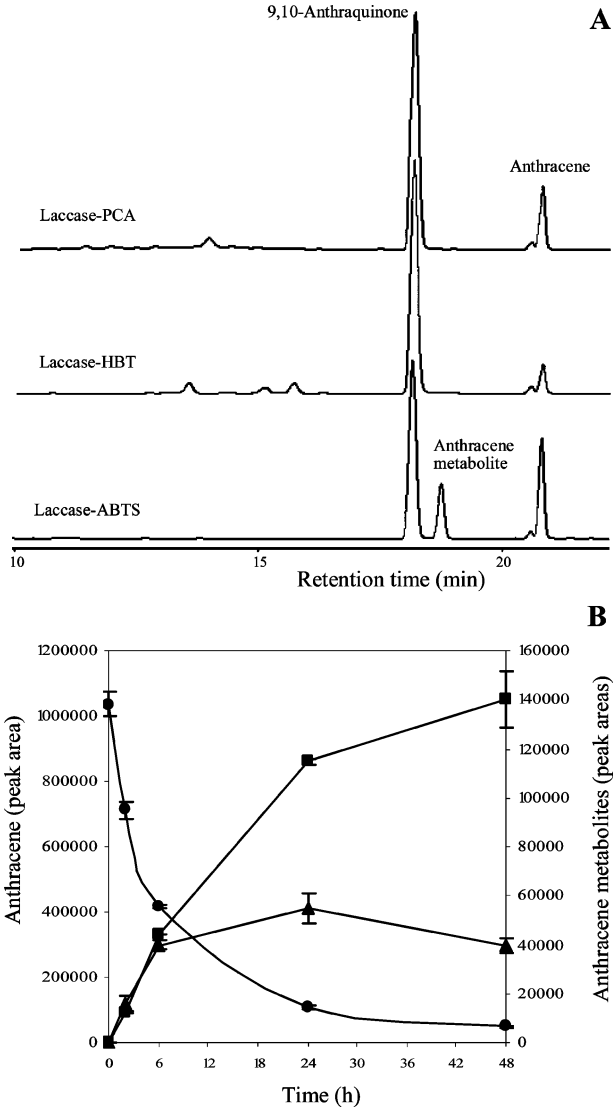

FIGURE 3. Comparison of HPLC profiles from 24 h-oxidation of anthracene (50 μM) with laccase and ABTS, HBT, or *p*-coumaric acid (PCA) (500 μM) showing the production of 9,10-anthraquinone and a second anthracene product when using laccase−ABTS (A) and time course of anthracene removal (●) and formation of 9,10-anthraquinone (■) and the unknown metabolite (▲) during anthracene oxidation with laccase−ABTS (B).

in a Pharmacia LKB 2248 system (VWM detector) using a Purospher STAR RP-18 endcapped column (5 μm, 4.6−250 mm) at 40 °C, with a flow rate of 1 mL/min, and a gradient from 80:20 0.1% acetic acid/acetonitrile to 100% acetonitrile (15 min) after 3 min of isocratic run. Absorption was monitored at 251 nm.

For detection of minor oxidation products, the reaction liquids were extracted with ethyl acetate, dried under N_2, and dissolved in 100 μL of acetonitrile prior to HPLC analysis using a photodiode array detector (PDA, Varian Prostar). 1,4-Dimethoxybenzene (500 μM) was added as internal standard. The ethyl acetate extracts were transferred to solvent-resistant polypropylene 96 well-plates, centrifuged for 5 min at 2200 g, sealed, introduced in a high-throughput HPLC autosampler (VWR, Hitachi L2200), and analyzed using a Nucleosil-C18 column (3 μm, 80 × 4 mm) at 15 °C. Acetonitrile−water (70:30 for anthracene reactions and 60:40 for benzo[a]pyrene reactions) was used as the mobile phase (1 mL/min flow rate).

Identification of Unknown Compounds from PAH Oxidation. The ethyl acetate extracts from the laccase−ABTS reactions were analyzed by HPLC/MS using a high-performance hybrid quadrupole time-of-flight (TOF) mass spectrometer (QSTAR XL Hybrid LC/MS/MS System, Applied Biosystems) with electrospray interface. Chromatographic separation (HPLC 1100, Agilent Technologies) was carried out under same running conditions of HPLC-PDA.

Electron impact spectra were obtained in a VG AutoSpec mass spectrometer with magnetic sector analyzer. Samples were introduced by direct probe at 200 °C and 70 eV energy.

Direct chemical ionization at atmospheric pressure with flow injection analysis (APCI-FIA) was carried out at different fragmentation energies (30, 60, 90, and 120 V), using acetonitrile−0.1% formic acid (50:50) as solvent at 0.2 mL/min flow, vaporizer temperature of 350 °C, and 45 psi nebulizer pressure.

Results

Anthracene Transformation. First, the mediating capabilities of syringaldehyde, acetosyringone, vanillin, acetovanillone, 2,4,6-trimethylphenol, and *p*-coumaric acid were evaluated during oxidation of anthracene by *P. cinnabarinus* laccase through colorimetric determination of the 9,10-anthraquinone produced (measured as 9,10-anthrahydroquinone). Among the natural compounds tested as mediators, *p*-coumaric acid produced the highest transformation of anthracene, similar to that attained using the synthetic mediator HBT.

Thereafter, the influence of mediator (*p*-coumaric acid) concentration on the anthracene oxidation rate was analyzed by HPLC. *P. cinnabarinus* laccase was able to transform 60% of the initial anthracene (50 μM) in 24 h without mediator,

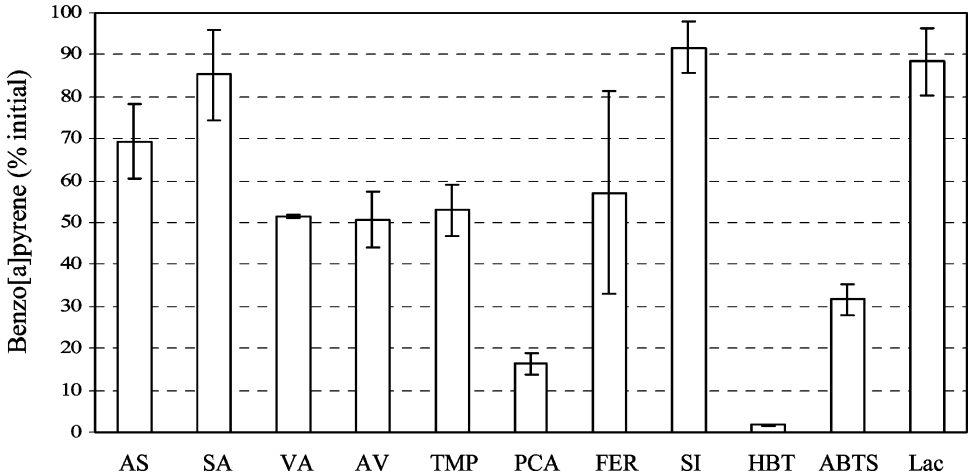

FIGURE 4. Residual benzo[a]pyrene (in % of initial 50 μM) after 24-h oxidation with laccase and different mediators (500 μM). Mean values and 95% confidence limits are shown. Mediators: acetosyringone (AS), syringaldehyde (SA), vanillin (VA), acetovanillone (AV), 2,4,6-trimethylphenol (TMP), *p*-coumaric acid (PCA), ferulic acid (FER), sinapic acid (SI), HBT, and ABTS (Lac, laccase without mediator).

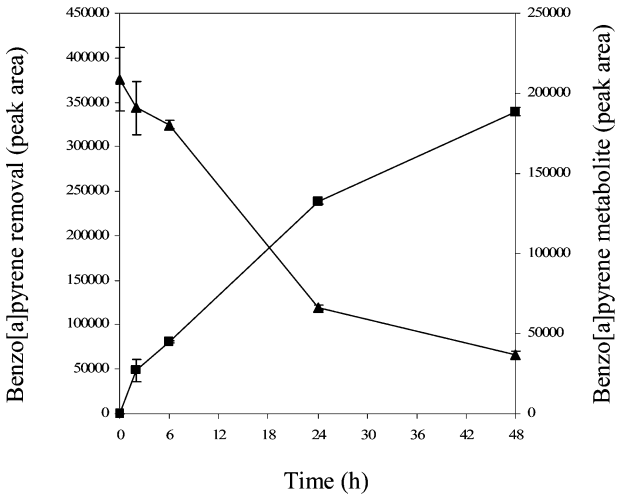

FIGURE 5. Removal of benzo[a]pyrene (50 μM) and formation of unknown metabolite by laccase and ABTS (500 μM). Mean values and 95% confidence limits are shown.

although only 9% was recovered as 9,10-anthraquinone. The presence of *p*-coumaric acid raised anthracene removal by laccase, but no significant improvement was obtained with concentrations over 100 μM (Figure 1A). By contrast, the yield of 9,10-anthraquinone formed was highly influenced by *p*-coumaric acid concentration. In fact, a direct correlation between the concentration of this mediator and the amount of 9,10-anthraquinone produced was observed (Figure 1B). Up to 50% of initial anthracene was recovered as 9,10-anthraquinone after 48 h in the presence of the highest concentration of *p*-coumaric acid. The amount of quinone decreased at longer incubation periods using high mediator concentrations. The same occurred when using HBT as laccase mediator (data not shown). However, no further oxidation or oligomerization products could be detected under the conditions assayed.

Comparison of anthracene oxidation rates obtained with laccase and the natural compounds vanillin, acetovanillone, syringaldehyde, acetosyringone, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, and sinapic acid and the synthetic mediators HBT and ABTS (using mediator/anthracene molar ratio of 10) is shown in Figure 2. 9,10-Anthraquinone was the main product detected from anthracene oxidation by all the laccase−mediator systems tested. In agreement with data shown above, *p*-coumaric acid resulted as the best natural mediator among the phenolic compounds assayed (followed by vanillin and acetovanillone). Neither ferulic acid nor sinapic acid behaved as good as a laccase mediator as *p*-coumaric acid did. In fact, sinapic acid did not promote the transformation of anthracene to 9,10-anthraquinone by laccase. *p*-Coumaric acid produced a higher and more rapid oxidation of anthracene than ABTS, quite similar to that obtained with HBT (95% in 24 h using laccase−PCA and 99.5% using laccase−HBT). At the same time, 36% and 52% 9,10-anthraquinone (molar %) were produced by laccase−PCA and laccase−HBT, respectively (Figure 2).

During oxidation of anthracene by laccase−ABTS a new product that could not be detected with any of the other laccase−mediator systems was found (Figure 3A). The amount of 9,10-anthraquinone increased up to 30% of the initial anthracene concentration throughout the 48 h-reaction, whereas the unknown product decreased after 24 h (Figure 3B) suggesting an earlier transformation by the laccase−ABTS system. UV−visible spectra of the unknown product and anthracene were very similar. HPLC/TOF−MS analysis of the reaction mixture showed an m/z 195 ion (M+1) that might correspond to the molecular mass of anthrone (9-oxoanthracene) or (9-hydroxyantracene), although other species seemed to elute in the same peak. Identification could not be confirmed by electron impact MS due to the complexity of the spectrum.

Benzo[a]pyrene Transformation. *P. cinnabarinus* laccase without mediator oxidized only 12% of benzo[a]pyrene in 24 h. The presence of the natural compounds vanillin, acetovanillone, 2,4,6-trimethylphenol, and, above all, *p*-coumaric acid notably promoted transformation of benzo[a]pyrene by laccase (mediator/PAH molar ratio of 10) (Figure 4).

No oxidation products were detected by direct HPLC analysis of the reaction mixtures except for the remarkable accumulation of an oxidation product during transformation

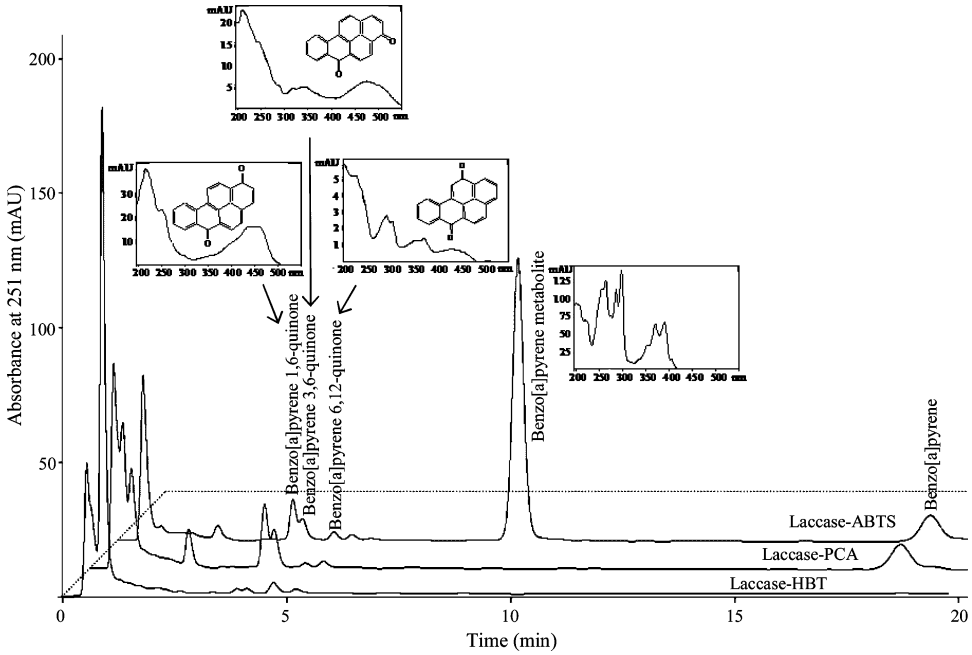

FIGURE 6. Comparison of HPLC profiles after oxidation of benzo[a]pyrene (50 μM) with laccase in the presence of ABTS, HBT, or *p*-coumaric acid (PCA) (500 μM) for 48 h. The UV−visible spectra from the three benzo[a]pyrene quinones produced by the enzymatic systems and the main oxidation product from the reaction with laccase−ABTS are shown.

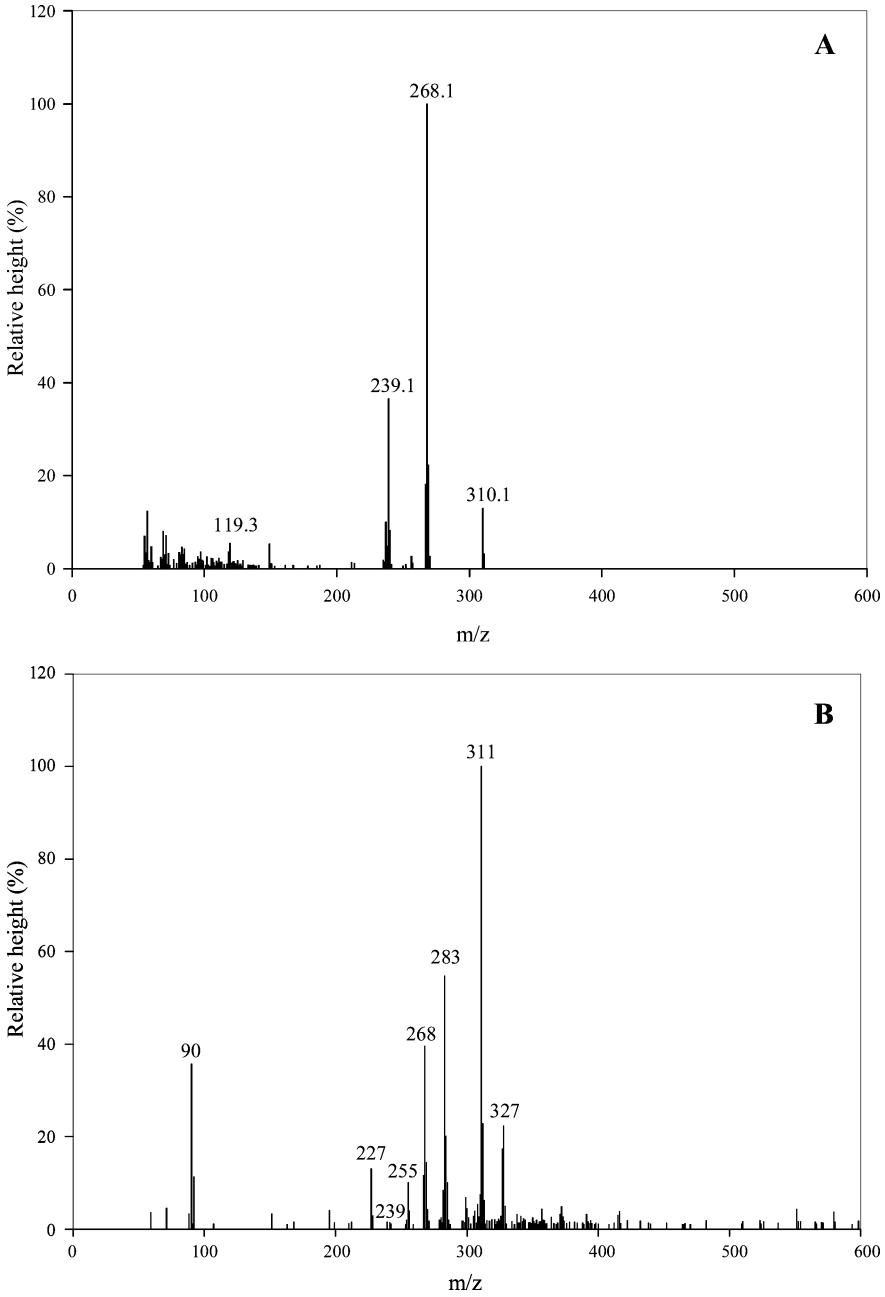

FIGURE 7. Mass spectra of the main product obtained from benzo[a]pyrene (50 μM) oxidation with laccase and ABTS (500 μM) after ethyl acetate extraction of the reaction mixture: (A) electron impact mass spectrum of the most volatile fraction of the unknown peak and (B) mass spectrum from APCI-FIA analysis of the same peak (30 v fragmentation energy).

of benzo[a]pyrene by laccase−ABTS (Figures 5 and 6). This product was not observed with any of the other laccase−mediator systems tested.

Extraction of samples with ethyl acetate and concentration prior to HPLC-PDA analysis enabled the detection and identification of minor products derived from the oxidation of benzo[a]pyrene with laccase and the selected natural mediator, *p*-coumaric acid, and the synthetic ones, HBT and ABTS. Three of these products showed the characteristic UV−visible spectra of benzo[a]pyrene 1,6-, 3,6-, and 6,12-quinones (Figure 6). No oxidation products were detected in the water soluble fractions, and acidification before extraction with ethyl acetate did not enable the detection of new oxidation products.

The UV−visible spectrum of the main product from benzo[a]pyrene oxidation with laccase−ABTS was very similar to that of benzo[a]pyrene. HPLC/TOF−MS analysis of the ethyl acetate extract of the reaction mixture (data not shown) showed an m/z 311 ion (M+1) in the unknown peak (although the presence of other chemical species was not discarded). Electron impact mass spectrum of the most volatile fraction of the unknown peak (Figure 7A) confirmed the presence of a product of 310 molecular mass. Formation of an acetate-derivative of benzo[a]pyrene (6-benzo[a]pyrenyl acetate) was supported by the molecular ion of m/z 310 and the base peak of m/z 268 consistent with the loss of the acetyl group yielding 6-hydroxybenzo[a]pyrene. Moreover, m/z 119 and 239 ions, characteristic of the mass spectra of hydroxybenzo[a]pyrene isomers, were also detected. Identification of 6-benzo[a]pyrenyl acetate as the main product from laccase−ABTS oxidation of benzo[a]pyrene was confirmed by APCI-FIA analysis, being m/z 311 the major ion (Figure 7B) that was more or less fragmented depending on the fragmentation energies applied. Nevertheless, together

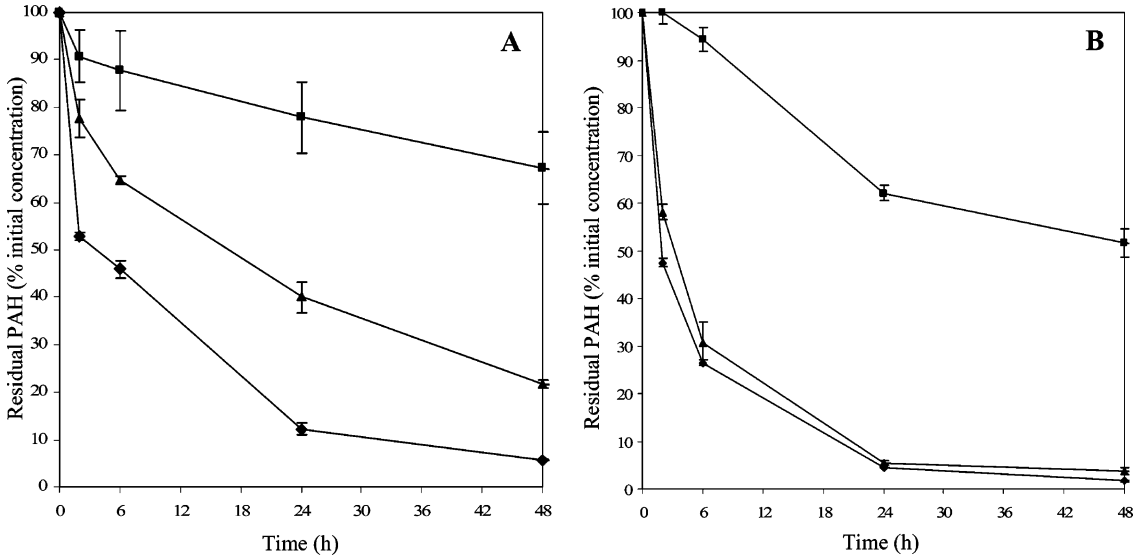

FIGURE 8. Comparative removal of anthracene (◆), benzo[a]pyrene (▲), and pyrene (■) (50 μM) by laccase−PCA, using two mediator concentrations 100 μM (A) and 1 mM (B). Mean values and 95% confidence limits are shown.

with the m/z ions specific for this metabolite, other ions that might correspond to a subsequent oxidation derivate such as hydroxy-6-benzo[a]pyrenyl acetate, with a molecular ion of m/z 327 (M+1) and fragmentation peaks of m/z 283 and 255, were also observed.

Comparative Transformation of PAHs by Laccase and *p*-Coumaric Acid. Oxidation of anthracene, benzo[a]pyrene, and pyrene by laccase−PCA were compared using two (low and high) mediator concentrations (Figure 8A,B). Pyrene was hardly degraded by *P. cinnabarinus* laccase without mediator (only around 8% in 24 h). The presence of *p*-coumaric acid increased pyrene removal as occurred for anthracene and benzo[a]pyrene. The oxidation rates of the most recalcitrant PAHs were more influenced by the concentration of mediator. Whereas almost no differences could be observed with respect to anthracene removal by laccase−PCA after 24 h, residual benzo[a]pyrene and pyrene decreased 35% and 20% (of the initial 50 μM), respectively, when *p*-coumaric acid concentration raised from 100 μM to 1 mM. Thus, almost complete removal of anthracene and benzo[a]pyrene and 50% transformation of pyrene were attained after 24-h reaction with laccase−PCA.

Discussion

We demonstrate here the capabilities of several naturally occurring phenols, namely vanillin, acetovanillone, 2,4,6-trimethylphenol, ferulic acid, and *p*-coumaric acid, to mediate transformation of PAHs by laccase. The outstanding efficiency of *p*-coumaric acid resulted in close similarity to that of the synthetic mediator HBT (*15*).

PAH Oxidation by Laccase−Mediator. Unlike other studies with natural mediators (*21*), the optimal removal of anthracene by laccase−PCA was attained with only twice as much of the mediator as of anthracene. On the contrary, higher mediator amounts notably increased the formation of 9,10-anthraquinone, since several oxidation steps are required to transform the PAH into its quinone. The percentages of 9,10-anthraquinone recovered (maximum 50% of the anthracene removed) together with the decrease of anthraquinone obtained at long incubation periods indicated that the quinone is not the end product of the reaction, and subsequent transformation by the laccase mediator system might have occurred. However, no polymerization products, nor oxidation products such as 2-(2′ hydroxybenzoyl)benzoic acid or phthalic acid, formed after subsequent oxidation, and ring opening of anthraquinone (*3*, *22*) could be detected under the conditions used. On the other hand, the possible identification as anthrone of the other product obtained with laccase−ABTS coincided with that described during anthracene oxidation by ligninolytic peroxidases previous to anthraquinone formation (*23*−*25*).

Electrochemical (*26*) and enzymatic oxidation of benzo[a]pyrene (*5*, *27*) generate benzo[a]pyrene quinones. Benzo[a]pyrene 1,6-, 3,6-, and 6,12-quinones were detected during oxidation of benzo[a]pyrene by *P. cinnabarinus* laccase and *p*-coumaric acid, HBT, or ABTS. Their presence as minor products would be in concordance with their condition as transient metabolites of the complete oxidation of PAH achieved by ligninolytic oxidorreductases as MnP (*28*), although subsequent oxidation products were not identified. On the other hand, the main product accumulating during transformation of benzo[a]pyrene by laccase−ABTS was not detected when sodium acetate buffer was replaced by sodium citrate. Thus, once oxidized by laccase−ABTS the radical cation of benzo[a]pyrene would be subjected to nucleophilic attack by the acetate anions present in the reaction mixture (*29*), producing the 6-benzo[a]pyrenyl acetate derivative, which was much more stable than the 6-hydroxybenzo[a]pyrene intermediate (*5*, *26*).

Reaction Mechanisms in PAH Oxidation by Laccase and Natural Mediators. Differences in the anthracene and benzo[a]pyrene oxidation products obtained with laccase−ABTS vs laccase−HBT or laccase−PCA upholds the dissimilarity of their oxidation mechanisms. ABTS-mediated reactions follow an electron transfer (ET) route, whereas HBT (nitroxyl) radicals oxidize the aromatic substrate by a hydrogen atom transfer (HAT) route (*30*). The *p*-coumaric acid phenoxyl radicals would act similarly to nitroxyl radicals, being the difference between dissociation energies of the bonds cleaved (C−H in the PAH) and formed (RO−H in the mediator) the driving force of this mechanism. The absence of the benzo[a]pyrenyl acetate intermediate would sustain the formation of PAH free radicals by laccase−PCA (and laccase−HBT). Moreover, the oxidation rates of anthracene, benzo[a]pyrene, and pyrene by laccase−PCA did not correlate with their ionization potentials (*15*).

The excellent performance of *p*-coumaric acid as laccase mediator is in concordance with data from other *p*-substituted phenols (*21*), and it had been also demonstrated during decolorization of organic dyes, together with acetosyringone and syringaldehyde (*17*). However, the two latter compounds that are promptly oxidized by the enzyme to stable phenoxyl

radicals (*31*), due to the presence of electron-donating groups, in *ortho* positions to the phenol group did not significantly promote PAH oxidation by laccase. The same occurred with the phenoxyl radicals of sinapic acid which is even faster oxidized by the enzyme. The catalytic efficiency of *P. cinnabarinus* laccase to oxidize sinapic, ferulic, and *p*-coumaric acid (3.9, 1.4, and 0.01 s^{-1} μM^{-1}, respectively) is inversely correlated with their capabilities to promote PAH oxidation, suggesting better mediating abilities for worse laccase substrates. Important issues as the balance between stability and reactivity of the oxidized species (phenoxyl radicals) are implicated (*32*). So far, fast coupling reactions among the free phenoxyl radicals (*33*) might be involved.

Role of Laccase and Natural Mediators. Laccases are ubiquitous enzymes in saprophytic fungi growing on soil and decayed plants (*34*). *p*-Hydroxycinnamic acids and lignin derived phenols, which are also present in soil, induce laccase expression by white-rot fungi (*19*, *35*). Phenolic compounds, originated from tree litter (*36*) and incorporated to humic substances, are also present in free form (*37*), being used as bioindicators of the ongoing process of humus formation (*38*). *p*-Coumaric acid and ferulic acid are abundant in forest litter (*39*) and in soils containing high contents of nonwoody plants (*40*), being also present in root exudates of trees (*41*). The successful in vitro transformation of PAH by laccase in the presence of the above phenolic compounds supports the potential role of these systems for the in vivo degradation of PAH in soil. Laccase-aided decontamination of these pollutants could be directed not only toward their immobilization (humification) by oxidative-coupling (*42*, *43*) but also toward their oxidative transformation (promoted by phenolic mediators) to easily up-taken products by soil microflora (*44*). Moreover, the use of environmentally friendly mediators easily available at low cost could smooth the progress of application of laccase−mediator systems in decontaminating or white-biotechnological industrial processes.

Acknowledgments

This work has been supported by the Spanish Programmes (CTQ2005-08925-C02-02/PPQ, VEM2004-08559, and BIO 2005-3569) and EU contract NMP2-CT-2006-026456. The authors thank Beldem (Belgium) for *P. cinnabarinus* laccase preparation and M. J. Vicente and R. Sedano (SIdI, Universidad Autónoma, Madrid) for HPLC/MS, EI, and APCI analyses. S.C. and M.A. thank R&C Contracts of the Spanish MEC, and A.I.C. thanks an I3P Fellowship of CSIC.

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Received for review September 29, 2006. Revised manuscript received January 29, 2007. Accepted February 2, 2007.

ES062328J