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Leila Qasemian, Christophe Billette, Daniel Guiral, Emilie Alazard, Magalie Moinard, et al.. Halotolerant laccases from *Chaetomium* sp., *Xylogone sphaerospora*, and *Coprinopsis* sp. isolated from a Mediterranean coastal area. *Fungal Biology*, 2012, 116 (10), pp.1090-1098. 10.1016/j.funbio.2012.08.002 . hal-02075881

HAL Id: hal-02075881

<https://hal.science/hal-02075881>

Submitted on 21 Mar 2019

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Halotolerant laccases from *Chaetomium* sp., *Xylogone sphaerospora*, and *Coprinopsis* sp. isolated from a Mediterranean coastal area

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ABSTRACT

Laccases (EC 1.10.3.2) are phenoloxidases involved in the transformation of the recalcitrant fraction of organic matter in soil. These enzymes are also able to transform certain aromatic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and are known to be inhibited by chloride ions. This study aims to test the potential of some fungal strains newly isolated from natural environments subjected to high osmotic pressure such as coastal ecosystems, to produce chloride tolerant laccases. Three strains were identified as *Chaetomium* sp., *Xylogone sphaerospora* (two Ascomycota), and *Coprinopsis* sp. (a Basidiomycota) and the laccases produced by these fungi were weakly inhibited by chloride ions compared with previous data from literature. Moreover, we tested their reactivity towards various PAHs which are widespread anthropic pollutants. They were able to transform anthracene to 9,10-anthraquinone and we determine 7.5 eV as the threshold of ionization potential for PAH oxidation by these laccases.

Keywords:

Anthropic pollutants
Chloride ions
Enzyme inhibition
Laccases
Osmotic pressure

Introduction

White-rot fungi are known to be involved in the transformation of the recalcitrant fraction of organic matter in soils and litters i.e. natural aromatic compounds derived from lignin. These fungi transform phenols via phenoloxidases such as laccases (*p*-diphenol: oxygen oxidoreductases, EC 1.10.3.2), and peroxidases like Mn-peroxidases (EC 1.11.1.13) or lignin-peroxidases (EC 1.11.1.14) (Thruston 1994; Baldrian 2006; Sinsabaugh 2010). This enzymatic system is also involved in ontogeny processes such as melanin-like pigment synthesis

in conidiospores or in the induction of fruiting bodies (Gómez *et al.* 2001; Frases *et al.* 2006; Xing *et al.* 2006; Montoya *et al.* 2012). Laccases as metalloenzymes (with several copper atoms in the active site) oxidize aromatic compounds while reducing oxygen to water. Since this reaction is not specific, laccases can also be involved in the transformation of aromatic pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Majeau *et al.* 2010), pesticides or dyes (Jaouani *et al.* 2005; Rodríguez-Couto *et al.* 2005; Riva 2006). Indeed, laccases are used in various industrial applications such as pulp bleaching (Fillat *et al.* 2010) or treatment of industrial effluents

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which sometimes involve media with high osmotic pressure (Rodakiewicz-Nowak 2000; Zeng et al. 2011). Because of the reaction they catalyze, these enzymes are indeed crucial both in recycling recalcitrant organic matter and in the detoxification of natural environments.

In a previous study, we tested the effects of various metal and halide salts on the laccases from *Marasmius quercophilus* and we found that certain laccase isoforms were actually strongly inhibited by chloride ions (Farnet et al. 2009). On the other hand, we found high laccase activities in a *Pinus halepensis* litter from Mediterranean coastal areas subjected to high osmotic pressure (Qasemian et al. 2011). Furthermore, coastal areas are known to be highly urbanized and thus chronically subjected to anthropic pollutants such as PAHs. These widespread pollutants are recalcitrant to biotransformation because of their aromatic and condensed structure leading to very low solubility (Muhit et al. 2008). This finding suggests that it would be useful to investigate how laccases produced by fungal strains isolated from such environments function in the presence of chloride ions and whether they are able to transform ubiquitous pollutants such as PAHs.

Here, we isolated and identified three fungal strains producing laccases from a *P. halepensis* litter in a coastal Mediterranean area (Marseillevyre, Bouches du Rhône, France) near the city of Marseilles (more than 850 000 inhabitants). Laccase activities were tested in the presence of chloride ions and we also examined their potential to transform various PAHs.

Materials and methods

Isolation of fungal strains from a litter of *Pinus halepensis*

A composite sampling was performed from partially degraded litter (horizon L) of Aleppo pine (*P. halepensis* L.) located on the French Mediterranean coast (Massif de Marseillevyre, Marseille). The salinity of the litter was monitored monthly over 1 y, by measuring the electrical conductivity (DiST 3 HANNA instruments) using litter extract, 1:10 w/v litter/H₂O. About 1 g of litter was spread onto plates of a malt extract agar medium (MEA), (malt extract, (Bio Mérieux, France): 20 g L⁻¹, agar (Bio Mérieux, France): 20 g L⁻¹) complemented with guaiacol 0.5 g L⁻¹ and supplied post autoclaving with chloramphenicol, (Sigma, France): 50 mg L⁻¹. This experiment was repeated 40 times. Fungal colonies which produced a purple halo were selected and used to inoculate a MEA medium to obtain pure mycelial cultures. Pure cultures on MEA medium were then used to prepare precultures.

Identification of the selected fungi by rDNA sequence analysis and reconstruction of their phylogenetic tree

The genomic DNA was extracted from the fungal cells using a Nucleon Phytopure extraction kit (Amersham Bioscience) from lyophilized mycelium cultured on MEA medium. The absorbance ratio A₂₆₀/A₂₈₀ was determined to confirm the purity of the template DNA. Two flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 (5'-TCCgTAggTgAACCTgCgg-3') and ALRO (5'-CATA-TgCTTAAgTTCAGCggg-3') (Collopy et al. 2001). β -tubulin

partial sequence was amplified using a Psathyrellaceae-specific primer pair (B36f psa 5'-CACCCACTCMCTYggTggTg-3' and B12r psa 5'-CATgAAGAAARTgRAGACgVgggAA-3'), modified from Thon & Royse (1999). The PCR mixture (25 μ L) contained 5 \times PCR buffer (Promega, Madison, WI), 5 μ L, dNTP mix (1.2 mM) (Eurobio, France), 3.5 μ L, primer (10 μ M), 2 μ L of each, template DNA (50 ng μ L⁻¹), 1 μ L, and GOTaq DNA polymerase 5 U (Promega M830B), 0.2 μ L. The reagents were combined in 500 μ L microtubes and placed in an Eppendorf Mastercycler (USA) programmed as follows: (a) initial denaturation at 95 °C for 5 min; (b) 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, and synthesis at 72 °C for 1 min; and (c) final extension at 72 °C for 4 min. For β -tubulin, PCR procedure followed those published by Nagy et al. (2011). Amplified DNA was separated on 1 % agarose gel in (EUROMEDEX, France) 0.5 \times Tris/Borate/EDTA (TBE) buffer for control. Sequencing was performed by Cogenics (Beckman Coulter genomics, United Kingdom). To compare the sequences of the amplified DNA with the sequences in GenBank, the basic local alignment tool (BLASTn) was used. The 666 bp (5.8S rDNA, ITS1, ITS2) sequences were submitted to GenBank under accession numbers JF681945, JF681946, and JF681944 respectively for CAL1, CAL2, and CAL3. RPB2 sequences were submitted to GenBank under accession numbers JX298877 and JX257156 respectively for CAL1 and CAL3. β -tubulin sequence for CAL2 was submitted under accession number JX218025. The phylogenetic trees were reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). First sequences were aligned with MUSCLE (v3.7) and then ambiguous regions were removed with Gblocks (v0.91b) with default settings. Partial sequence was used for β -tubulin gene. The first intron present in *Coprinopsis cinerea* and absent in all other sequences, has been removed. The second intron, present in all sequences, has been conserved in the analysis. In ML analysis, the default substitution model was selected assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for heterogeneity rate across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (500 bootstrap replicates). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

Laccase production in liquid culture and optimum pH determination

For each strain, precultures were performed in 200 mL Erlenmeyer flasks with 50 mL of Malt extract liquid medium (MEL) containing malt extract (20 g L⁻¹) and CuSO₄ (5 g L⁻¹). They were inoculated with a plug (1 cm diameter) from a fungal culture grown on MEA and incubated at 25 °C in darkness for 4 d. These precultures were used to inoculate 1 L-Erlenmeyer flasks with 200 mL of MEL. Cultures were incubated at 25 °C for 5 d in darkness under shaking at 200 rpm (three replicates per strain).

Enzyme activity was measured by following the oxidation of syringaldazine [N,N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] to quinone ($\epsilon^M = 65\ 000\ \text{M}^{-1}\ \text{cm}^{-1}$) at 525 nm in acetate buffer 0.1 M, pH 4.0 on a Kontron Uvikon 860

spectrophotometer. One unit (U) of laccase activity is defined as the amount of enzyme that oxidizes 1 μmol of the substrate min^{-1} . Culture sampling (two replicates) was performed during one week (each day) in order to determine the incubation time where the maximum laccase activity was obtained. The reaction mixture contained 500 μL of the medium, 10 μL of syringaldazine (0.6 % in methanol), and 2.5 mL of acetate buffer (two replicates for each sampling). According to the best laccase production, cultures were filtered on a glass microfibre filter GF/D, 2.7 μm (Whatman, England). The filtered medium was concentrated using dialysis tubes (Cellu Sep, VWR, France) rated at 10 Molecular Weight (10 kD) cut-off. A total volume of 10 mL was obtained. Optimum pH was determined using 15 μL of the concentrated fraction with 10 μL of syringaldazine s.q.f. 1 mL of the adequate buffer. Acetate buffer 100 mM was used for the pH range from 4 to 5 and phosphate buffer 100 mM for the pH range from 5.7 to 8. The optimum pH was used for further activity measurements.

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF)

Laccase isoform patterns were obtained from a PAGE carried out using 4 % stacking gel and 10 % separating gel at 220 V with the Mini-Protean II electrophoresis cell (Biorad). Laccase isoforms were stained using *p*-phenylenediamine 0.1 % in acetate buffer 100 mM pH 4.5 at 30 °C under gentle shaking.

IEF was performed using the Biorad Rotofor preparative Cell. The samples were prepared as follow: 1 mL of Bio-Lyte 3/10 ampholytes (Biorad) was added to 10 mL of the enzyme extract and distilled water s.q.f. 50 mL. IEF was performed for 3 h at 200 V until the pH gradient become stable (voltage about 360 V). 2 mL-fractions were collected under vacuum and pH was immediately measured for each fraction. Laccase activity was also measured for each fraction using syringaldazine as a substrate as described above. For each strain, the fraction exhibiting the higher laccase activity was used to test the effect of chloride ions.

Effect of chloride ions on isolate growth and their laccase activities

The effects of chloride ions on each strain growth were tested by following the daily growth on solid cultures (as explained in 2.1) supplied with 0, 2.5, 5, 10, 20 g L^{-1} of NaCl. To test halotolerance of laccases, the IEF fraction with the highest activity was used. Syringaldazine was used as substrate to determine the V_m and K_m , which were calculated using the Lineweaver–Burk transformation of the Michaelis–Menten equation. These kinetic parameters were calculated with and without NaCl in the reaction mixture. The concentrations ranged from 12.5 to 75 μM for syringaldazine, two concentrations, 0.1 and 0.2 M, were used for NaCl in acetate buffer 0.1 M pH 4.5 and 5 U of laccase activities was used for each experiment.

PAH detection by high performance liquid chromatograph (HPLC)

The HPLC system was equipped with a C18 Reverse Phase column (Merck, 4.6 \times 250 mm) in the following gradient system:

solvent A, 90/10 (v/v) of 0.1 % water–trifluoro acetic acid/acetonitrile, solvent B, 95/5 (v/v) of 0.1 % water–trifluoro acetic acid/acetonitrile, gradient = 5–15 min, A 100 % to B 100 %; 15–25 min B 100 % (flow rate 1 mL min^{-1}). The PAH transformation was realized using the IEF fraction with the highest activity from the three strains. The reaction mixture (5 mL) contained 250 mg L^{-1} PAH and 5 U of laccase in acetate buffer 0.1 M pH 4.5 and 10 % acetone. The reaction mixtures were incubated at 30 °C for 6 h in the dark. An extraction with 5 mL of dichloromethane was performed for each reaction mixture prior to injection. The organic phase was evaporated and the products of extraction were dissolved in 1 mL of acetonitrile (30 μL injected). The PAHs used were: anthracene, 9-methylanthracene, pyrene, perylene, and fluorene. All the PAHs were purchased from Sigma. *P*-hydroxybenzoic acid was used as reference in each injection. 9,10-anthraquinone was also injected in order to confirm the identification of the single oxidative product obtained after anthracene oxidation. Peaks were identified based on matching retention time and UV spectra ranging from 200 to 500 nm with those of anthracene and anthraquinone standards.

Results

Light microscopy examinations of the hyphae of the three strains are shown in Fig 1. Using sequences of ITS1 and ITS2, 5.8S rDNA, β -tubulin, and RPB2, strains CAL1 and CAL3 were identified as Ascomycota, respectively *Chaetomium* sp. and *Xylogone sphaerospora* and strain CAL2 was identified as a Basidiomycota, *Coprinopsis* sp. Based on ribosomal sequences, we then designed the phylogenetic trees of all isolates (Fig 2). ITS sequence of CAL1 has only two gap differences with HQ607809 and HQ607837 identified as *Chaetomium* sp. isolated from *Atta texana* in Central Texas (USA) (Rodrigues et al. 2011) and four gap differences with GQ176270, an endophytic fungus of *Tylophora indica* found in India (Fig 2A) (Kumar et al. 2011). CAL2 ribosomal gene sequence has 99 % identity and no gap difference with the 594 bp sequence (JF907840) of the *Coprinopsis spilospora* strain found in Italy (Fig 2B). CAL3 ribosomal gene sequences has 99 % identity and only two gaps differences with the 450 bp sequence (GQ272626) of the well-characterized *X. sphaerospora* strain KACC 41222 (Kang et al. 2010) found on preservative-treated timber. It has 99 % identity and three gaps differences with the 451 bp sequence (FJ362272) of an uncultured root-associated fungus found on *Yucca glauca* in New Mexico. It has 98 % identity and one gap difference with the 448 bp sequence (FN397286) of an uncultured fungus found in a *Tuber melanosporum* truffle-ground soil, in a burnt area, in Cahors (France). All these *X. sphaerospora* and those uncultivated and unidentified fungi sequences cluster in a single clade in the ITS phylogenetic tree (Fig 2C). β -tubulin and RPB2 genes were also sequenced to confirm our results. CAL2 β -tubulin sequence has 99 % identity and no gap difference with the 437 bp sequence (HQ847194) of *Coprinopsis alcobae*. CAL2 sequence clusters with those of *Coprinopsis spilospora*, *C. alcobae*, and *Coprinopsis subdomesticus*, in a well-characterized clade with a bootstraps value of 72 % for β -tubulin phylogenetic tree (data not shown). *Coprinopsis spilospora* is a well-known species since 1951, ITS of different strains have been sequenced. The two other species have been described more recently and

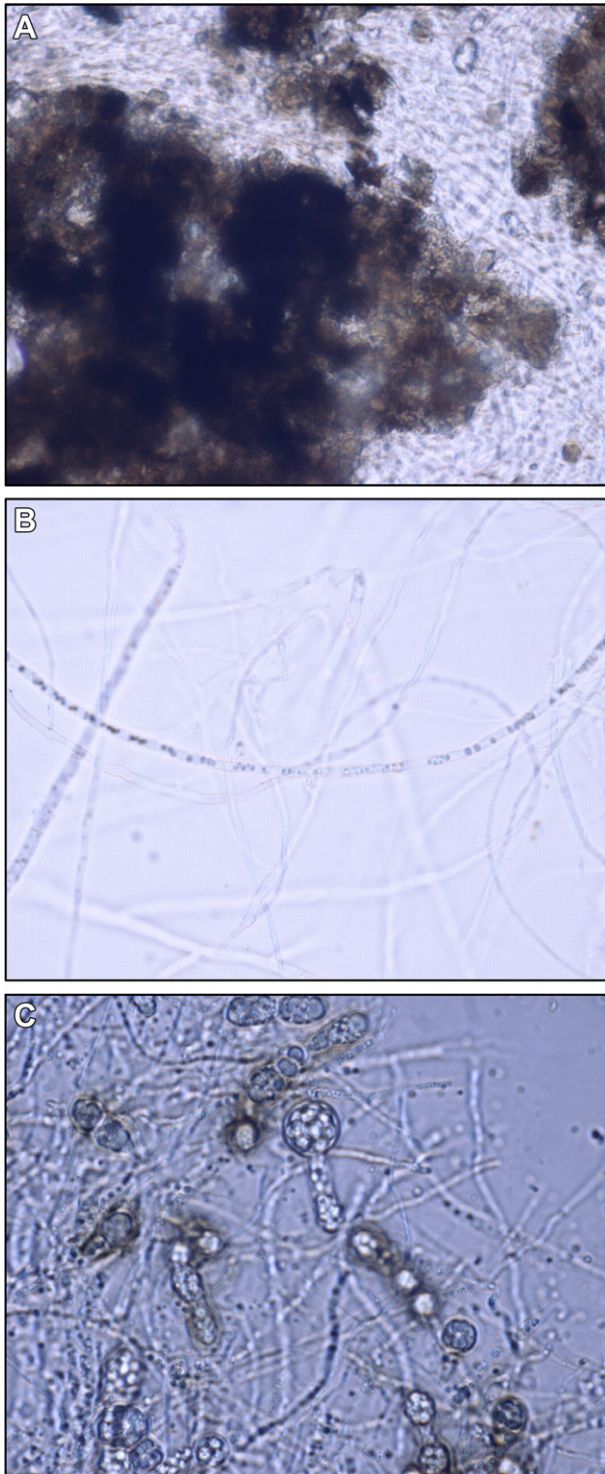


Fig 1 – Light microscopy examinations of isolates (A) *Chaetomium* sp. (B) *Coprinopsis* sp., and (C) *Xylogone sphaerospora*.

sequences of only one strain of each are present in GenBank. It is not possible to clearly identify CAL2 but it belongs to one of these three species. Using RPB2 gene the result is clearer since *X. sphaerospora* and CAL3 sequences cluster in a clade with a 100 bootstraps value and form a sister clade with *Scytalidium cuboideum* strains (data not shown). Thus CAL3 is identified as *X. sphaerospora*.

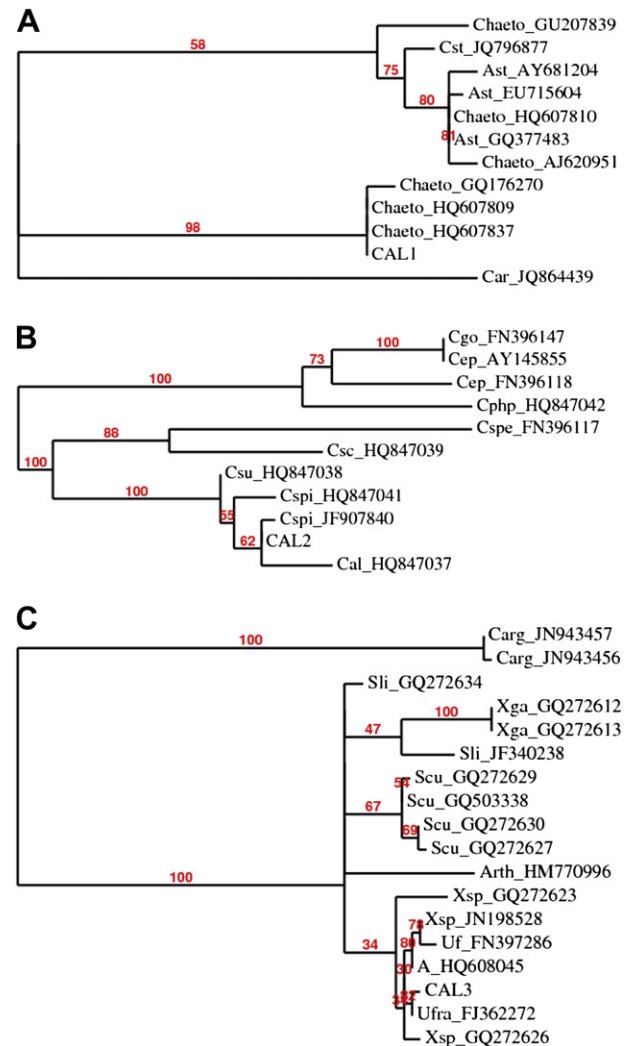


Fig 2 – Phylogenetic tree obtained from PhyML analysis based on ITS sequences for (A) *Chaetomium* sp. (CAL1), rooted with *Chaetomidium arxii* strain CCTU 275, species abbreviations: Chaeto, *Chaetomium* sp.; Cst, *Chaetomium strumarium*; Cgl, *Chaetomium globosum*; Ast, *Achaetomium strumarium*; Tco, *Thielavia coactilis*; Tfr, *Thielavia fragilis*; Cku, *Corynascus kuwaitiensis*; Clu, *Chaetomium luteum*; Car, *Chaetomidium arxii*, (B) *Coprinopsis* sp. (CAL2), species abbreviations: Cal, *Coprinus alcobae*; Csu, *Coprinus subdomesticus*; Cspi, *Coprinopsis pilospora*; Csc, *Coprinopsis sclerotiorum*; Cgo, *Coprinopsis gonophylla*; Cphp, *Coprinus phaeopunctatus*; Ccin, *Coprinopsis cinerea*; Cspe, *Coprinopsis spelaiophila*; Cep, *Coprinopsis episcopalalis*; Cphs, *Coprinopsis phaeospora*, and (C) *Xylogone sphaerospora* (CAL3) rooted with *Chlorociboria argentinensis*, species abbreviations: Uf, Uncultured fungus; Ufra, Uncultured root-associated fungus; Xsp, *Xylogone sphaerospora*; A, *Ascomycota* sp., Arth, *Arthrographis* sp.; Sli, *Scytalidium lignicola*; Scu, *Scytalidium cuboideum*; Xga, *Xylogone ganoder-mophthora*; Carg, *Chlorociboria argentinensis*. *Arthrographis cuboidea* has been reclassified as *Scytalidium cuboideum* by Kang et al. (2010). The anamorph of *X. sphaerospora* is named *Scytalidium sphaerosporum*. Branches with bootstrap values smaller than 50 % are collapsed except for C (bootstrap values smaller than 30 % are collapsed). Bootstrap values above 50 % and 30 % are shown.

First, we examined the optimum pH for laccase activity using syringaldazine as substrate (Fig 3). We obtained the maximum laccase activities for the three strains after 6 d in liquid cultures. Whatever the strain considered, optimum pH was 4.5 and we used this pH for all further activity measurements. This result corroborates previous studies: most laccases are active at low pH (Baldrian 2006).

Then, laccase isoform diversity expressed by these strains was determined using electrophoretic methods. Fig 4 shows the PAGE which revealed different profiles depending on the studied strains: two laccase isoforms ($R_f = 0.41$ and 0.5) for *Chaetomium* sp. strain, one isoform ($R_f = 0.29$) for *Coprinopsis* sp. strain, and one isoform ($R_f = 0.22$) for *X. sphaerospora* strain.

Preparative IEF was also performed: for *Chaetomium* sp. strain, four isoforms with pHi 3.5, 4, 4.6, and 5.7 were found, for *Coprinopsis* sp. strain, two isoforms with pHi 3.2 and 3.5 and for *X. sphaerospora* strain, one isoform with pHi 4.3. Of all fractions, the highest activity was obtained for the isoform with pHi 4 for *Chaetomium* sp. strain and for the isoform with pHi 3.5 for *Coprinopsis* sp. strain and we used the partially purified fraction with the highest laccase activity to test laccase halotolerance and activity in the presence of solvents.

The halotolerance of isolates was tested by comparing their growth on solid culture supplied with NaCl as ions representative of the main salts in sea water sprays (Fig 5A). Salinity of the sampling litter ranged from 300 to 1500 $\mu\text{S}\cdot\text{cm}^{-1}$ (from 192 to 960 ppm of Total Dissolved Solids, TDS) over a 1 y field measurement with a mean value of 778 $\mu\text{S}\cdot\text{cm}^{-1}$ (498 ppm TDS).

All three strains showed a rapid growth in solid culture based on malt extract without NaCl (3.8 mm d^{-1} for *Chaetomium* sp. and 5.1 for *Coprinopsis* sp. and *X. sphaerospora*). For all isolates, no growth was observed after 170 mM of NaCl in solid culture. Fig 5B shows the results of laccase activity obtained for NaCl concentrations ranging from 50 to 800 mM. Laccases from *Chaetomium* sp. strain were the most sensitive to chloride ions with 50 % of the laccase activities remaining (Inhibition Concentration 50, IC_{50}) at 100 mM NaCl. With laccases from *Coprinopsis* sp. strain and *X. sphaerospora* strain, IC_{50} was obtained for 150 and 200 mM, respectively. To clearly define the type of inhibition by chloride ions on the studied

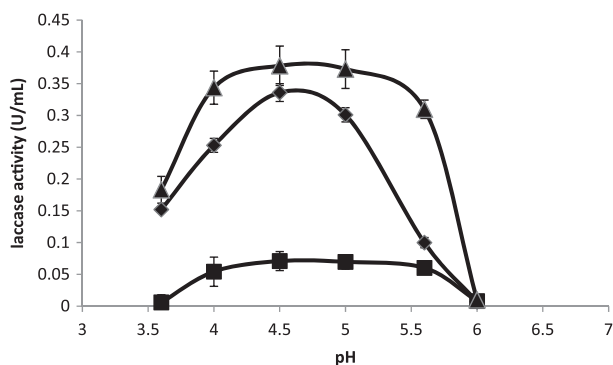


Fig 3 – Determination of optimum pH using the concentrated culture medium. Laccase activity was measured using syringaldazine as substrate for *Chaetomium* sp. (■), *Coprinopsis* sp. (◆), and *Xylogone sphaerospora* (▲).

Coprinopsis sp. *X. sphaerospora* *Chaetomium* sp.

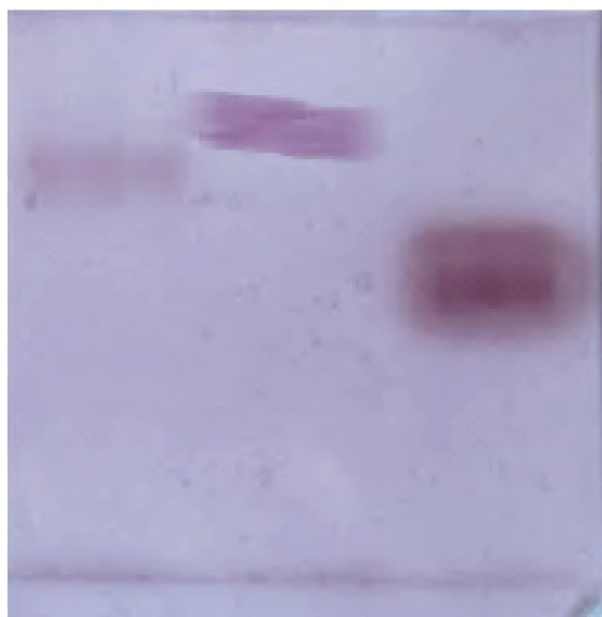


Fig 4 – PAGE pattern of laccases produced by *Coprinopsis* sp. ($R_f = 0.29$), *Xylogone sphaerospora* ($R_f = 0.22$), and *Chaetomium* sp. ($R_f = 0.41$ and 0.50) from left to right. The bands are revealed by laccase activity using *p*-phenylenediamine.

laccases, we measured laccase activities with various concentrations of substrate (syringaldazine) with 0.1 or 0.2 M of NaCl in the reaction mixture using the Lineweaver–Burk representation. The results obtained are shown in Fig 6 and demonstrate that chloride ions act as competitive inhibitors: the V_m remains constant while K_m decreases when the inhibitor is added. K_m for syringaldazine was 57, 76.4, and 57 μM and V_m 0.99, 1.47, and 2.15 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ for *Chaetomium* sp. strain, *Coprinopsis* sp. strain, and *X. sphaerospora* strain, respectively.

The effect of different solvents on laccase activities of the three strains was tested in order to choose the least inhibiting solvent, which would then be used to solubilize PAHs. Fig 7 shows that dioxane rapidly inhibits laccase activities for all the strains: no laccase activity remains with 10 % of dioxane in the reaction medium. Solvent inhibition in decreasing order is as follows: dioxane, acetone, methanol, and ethanol. We also found that laccase activities increase (more than 100 % of the initial activity) for weak concentrations of ethanol and methanol (up to 10 %).

Since acetone did not drastically alter laccase activity and the studied PAHs can be dissolved in this solvent, we used 10 % of acetone for the rest of our experiments in the reaction medium: about 90 %, 80 %, and 70 % of laccase activity remains for *Chaetomium* sp., *Coprinopsis* sp., and *X. sphaerospora* strains, respectively.

The potential for oxidation of PAHs by the three strains was studied using anthracene, 9-methylanthracene, pyrene, perylene, and fluorene. These PAHs were chosen according to their Ionization Potential (IP), respectively 7.43, 7.5, 7.6, 7.76, and 7.96 eV. For all the three strains, only anthracene was oxidized and this oxidation yielded 9,10-anthraquinone, according to the HPLC profile (data not shown).

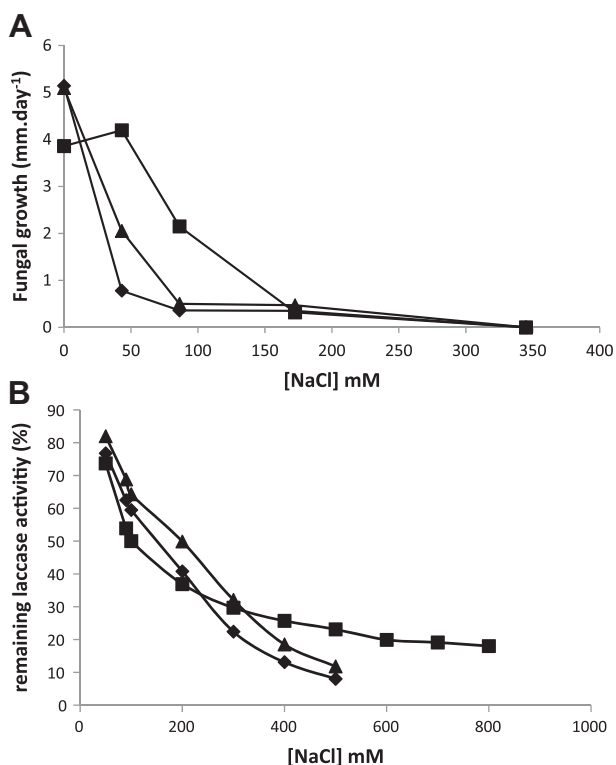


Fig 5 – Fungal growth on solid culture (A) and laccase activity produced in liquid culture (expressed as percentages of the remaining activity after NaCl addition) (B), with different concentrations of NaCl for *Chaetomium* sp. (■), *Coprinopsis* sp. (◆), and *Xylogone sphaerospora* (▲).

Discussion

In this study we tested the effect of chloride ions on the laccases of three fungal strains isolated from a Mediterranean coastal environment. These phenoloxidase activities have previously been proved to be inhibited by chloride ions for certain fungal species (Farnet et al. 2008; Bonugli-Santos et al. 2010). Our aim here was to demonstrate that areas naturally exposed to high osmotic pressure via sea sprays can be colonized by fungi producing laccases that remain active in the presence of such ions.

We used NaCl to rule out interference with the cation on laccase activity: we have previously shown that certain metallic ions such as Cu^{2+} or Fe^{2+} react with enzymes leading to an apparent laccase activity increase (Farnet et al. 2008).

Here, the Lineweaver–Burk transformations of Michaelis–Menten equation showed that chloride ions act as competitive inhibitors for the laccase under study. Various previous studies have described the inhibition of halide ions from an electrochemical point of view (Xu et al. 1998; Schneider et al. 1999): fluoride ions act as noncompetitive inhibitors by blocking the intramolecular electron transfer via binding to the T2/T3 copper cluster of laccase. On the other hand, chloride ions act as competitive inhibitors by blocking the access to the electron donor i.e. the aromatic compound to the copper of the T1 site. Vaz-Dominguez et al. (2008) corroborated these results using a laccase electrode, showing the different modes of laccase inhibition by halide ions.

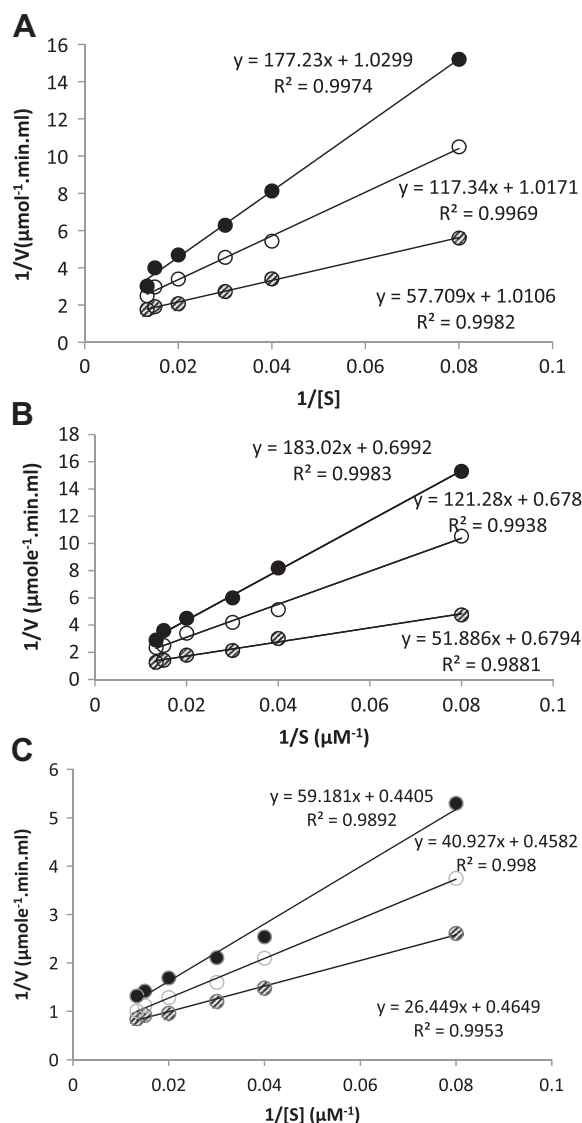


Fig 6 – Determination of the type of inhibition of chloride ions on laccase activity. Lineweaver–Burk transformations of Michaelis–Menten equation is represented for *Chaetomium* sp. (A), *Coprinopsis* sp. (B), and *Xylogone sphaerospora* (C) with NaCl 0.1 M (○), 0.2 M (●) and without NaCl (⊙).

It is noteworthy that these fungal strains produce laccases which are inhibited to a lesser extent than those expressed by other fungi such as the white-rot basidiomycete *Marasmius quercophilus* (Farnet et al. 2008), whose laccases were strongly inhibited with 10 mM of NaCl (no residual activity for this concentration). Furthermore, Kim & Nicell (2006) found a two-fold decrease in the oxidation of bisphenol A by a laccase from *Trametes versicolor* with 50 mM of NaCl. Up to now, ‘halotolerant’ laccases have been found mainly in bacteria isolated from particular natural environments: Actinomycetes such as *Streptomyces psammoticus* (Niladevi et al. 2008) or marine bacteria such as *Marinomonas mediterranea* (Jimenez-Juarez et al. 2005). Laccases isolated from these bacteria show very high tolerance to chloride ions: enzyme activities remain at concentrations of NaCl up to 1 and 1.2 M respectively. It is

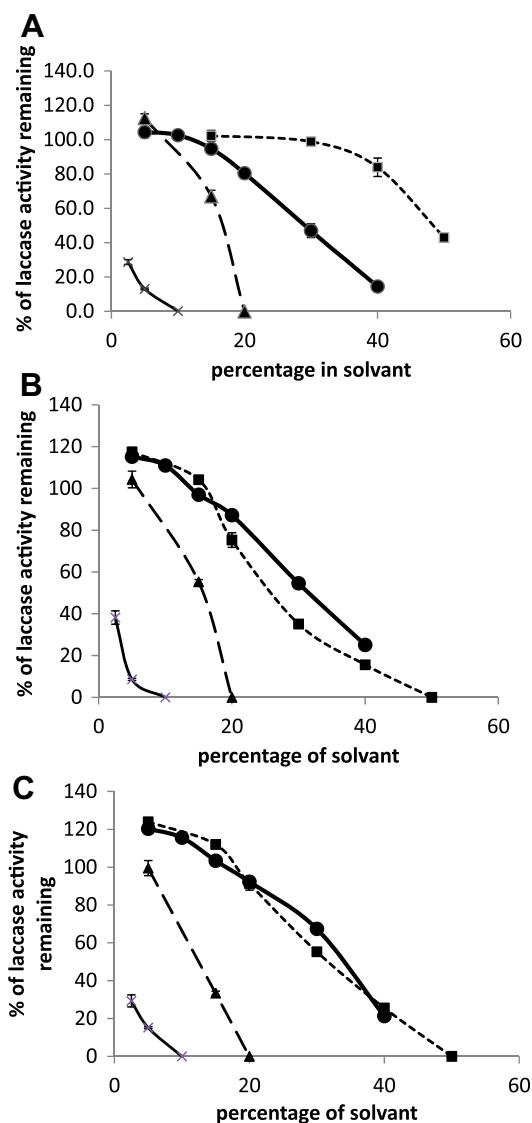


Fig 7 – Effect of solvents on laccase activities of the three isolates *Chaetomium* sp. (A), *Coprinopsis* sp. (B), *Xylogone sphaerospora* (C) expressed as percentages of activities remaining. The solvents used were: dioxane (—), methanol (- - -), ethanol (—▲), acetone (- - ×).

noteworthy that both bacteria were isolated from natural media with high osmotic pressure: *M. mediterranea* is a marine bacteria and *S. psammoticus* was isolated from a mangrove swamp. In contrast, laccases from bacteria belonging to Rhizobiaceae such as *Sinorhizobium meliloti* (Rosconi et al. 2005) are strongly inhibited by halides (NaF or NaCl). Here, the laccases produced by three fungal species *Chaetomium*, *Xylogone sphaerospora*, and *Coprinopsis* were still active for 100 mM of NaCl. Two of them belong to Ascomycota: several studies have shown that this fungal phylum is frequently found in saline and hypersaline environment (Bergbauer & Newell 1992; Raghukumar 2004; Gunde-Cimerman et al. 2009). Lyons et al. (2003) showed high diversity in laccase gene sequences in a study on salt marsh Ascomycota. Liers et al. (2007) biochemically described the laccases produced by an Ascomycota

Xylaria polymorpha which was not isolated from a saline environment and found that 20 % of the laccase activity remains with 1.2 M of NaCl. Other studies have also shown that a marine Basidiomycota produces halotolerant laccases (D'souza et al. 2006). Here we also observed that laccase halotolerance of the different isolates were not exactly correlated to the effect of NaCl on isolate growth on solid culture. Moreover the range of NaCl concentrations used to test halotolerance of the isolates (Fig 5A and B) were nearly 20 fold higher than the average value of litter salinity obtained in the sampling site. Thus, the isolates were tolerant to salinity concentrations higher than those measured in situ, suggesting that they are adapted to environmental salinity conditions. Thus it would be valuable to investigate the functional role of Basidiomycota and Ascomycota in phenolic compound oxidation in natural environments subjected to high osmotic pressure.

Laccase encoding-gene in the genus *Coprinopsis* has already been reported: Kilaru et al. (2006) described 17 laccase genes in the fungus *Coprinopsis cinerea*. Interestingly, we have recently found that peptides of laccases produced in litter from the same area can be assigned to laccases of *C. cinerea* species (Qasemian et al. 2011). The identification of a species from this genus thus corroborates our previous findings and indicates that *Coprinopsis* species are involved in laccase production in this type of natural environment. Laccase proteins and laccase genes (Lyons et al. 2003) have also been identified from Ascomycota such as *Penicillium chrysogenum* (Rodríguez et al. 1996) or *Aspergillus nidulans* (Scherer & Fischer 1998). Moreover, laccases produced by *Chaetomium* sp. have been already reported for *Chaetomium thermophilum* (Chefetz et al. 1998) while, to our knowledge, this is the first report of laccase produced by *X. sphaerospora*.

We focused on PAH transformation since these molecules are known to be ubiquitous environmental pollutants and have been detected in the studied area (Qasemian et al. 2012). Previous *in vitro* and *in situ* studies have shown that fungal phenoloxidases can be responsible for oxidation of PAHs (Canãs et al. 2007; Li et al. 2010). Laccases can thus play a major role in the detoxification of such natural ecosystems. Moreover, we have shown that high concentrations of chloride ions do not strongly affect the laccase activities of the fungal species isolated from coastal areas, suggesting that these enzymes are still active under these conditions associated with high osmotic pressure. This highlights the importance of this enzymatic system which is involved in the oxidation of both the recalcitrant fraction of soil organic matter and certain aromatic pollutants.

A previous study on the phenoloxidases from the white-rot fungus *M. quercophilus* (Farnet et al. 2009) showed that the ability of laccases to oxidize these molecules depended on their IP, and that molecules with IP over 8.1 eV were not oxidized. However the PAHs tested in this previous study were anthracene, naphthalene, and phenanthrene with IP of 7.43, 8.1, and 8.19 eV, and our aim here was to define a more accurate threshold of IP starting from which the laccases under study oxidize aromatic compounds.

Here, our study reveals a threshold for PAH oxidation at 7.43 eV ionization potential for these laccases which is in agreement with previous studies (Hammel 1995; Kay-Shoemaker & Watwood 1996; Dodor et al. 2004; Farnet et al.

2009). This result is of particular importance since this IP threshold can be used to forecast other molecules which can be oxidized by these laccases such as dyes or chlorophenol compounds for further biotechnological applications. Thus, when ionization potentials are available, they can be used to forecast the ability of a laccase to oxidize a molecule.

Mediterranean coastal environments are known to be hot spots of biodiversity. This study confirms our previous findings (Qasemian *et al.* 2011) showing that this environment can be a potential source of fungal species producing halotolerant laccases. Isolating new fungal strains with such biochemical features holds great promise for biotechnological development in such fields.

Acknowledgment

This work is financially supported by a PhD grant of the French Environment and Energy Management Agency (ADEME) and the Région Provence Alpes Côtés d'Azur (France). We thank Florence Ruauudel for her technical assistance and Marjorie Sweetko for English language improvement.

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