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Degradation pathway of pentachlorophenol by *Mucor plumbeus* involves phase II conjugation and oxidation–reduction reactions

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ABSTRACT

Environmental pollution by pentachlorophenol (PCP) is a critical concern worldwide and fungal bioremediation constitutes an elegant and environment-friendly solution. Mucorales from the Zygomycota phylum are often observed to be competitive in field conditions and *Mucor plumbeus*, in particular, can efficiently deplete PCP from media. The pathway for PCP degradation used by this fungus has not been investigated. In this study, PCP-derived metabolites were identified by liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, including tetra- and tri-chlorohydroquinones and phase II-conjugated metabolites. Amongst the latter are the previously reported glucose, sulfate and ribose conjugates, and identified for the first time in fungi sulfate–glucose conjugates. A PCP transformation pathway for *M. plumbeus* is proposed, which excludes the involvement of cytochrome P-450 and extracellular ligninolytic enzymes.

1. Introduction

Pentachlorophenol (PCP) was introduced in the environment in the 1930s [1]. Since then, it has been used in a variety of applications, mostly as a biocide. Due to concerns relating to its toxicity and recalcitrance to degradation, PCP has been recognised as a priority pollutant and its use worldwide has been severely restricted [2,3]. Extensive past use and renewed use in rapidly developing economies, together with its ability for long-range atmospheric transport, led to wide spread contamination of soils, sediments and waters [2,4,5]. PCP can also be formed as a by-product in water chlorination processes and as a metabolite from the degradation of some pesticides [1,2]. It is recognised as highly toxic, it uncouples oxidative phosphorylation, and has been described as an endocrine disruptor and a probable carcinogen [6–9].

The recalcitrance of PCP to degradation hampers its removal from the environment. Volatilisation and photolysis are usually slow [1]. In addition, incineration and stockpiling are expensive and inefficient strategies which may lead to the production of hazardous by-products [10]. Bioremediation, *i.e.* the use of microorganisms, plants or biologically active agents to degrade, sequester or conjugate environmental pollutants [11], is a clean and low-cost methodology [12]. Fungal bioremediation continues to attract interest, due primarily to the high diversity of species, their broad enzymatic capacities and high hyphal extension [11,13]. Basidiomycota fungi have the ability to produce lignin modifying enzymes, such as lignin peroxidase (LiP), Mn-dependent peroxidase (MnP) and laccase [14], and are able to transform a wide range of recalcitrant organopollutants [15]. The Basidiomycota *Phanerochaete chrysosporium* is the most extensively investigated for PCP degradation [16–20]. The PCP-derived metabolites and the degradation pathway have been elucidated [16]. However, this fungus like many others of its phylum, demonstrates low bioremediation efficiency in field conditions [14].

Zygomycota fungi, although less studied, are frequently isolated from environments contaminated with organochlorine compounds (*e.g.* [21–23]) and their ability to transform chlorinated phenols has been demonstrated (*e.g.* [24–28]). Amongst Zygomycota fungi, the resistance and capacity of *Mucor* spp. to transform complex xenobiotics has been highlighted (*e.g.* [29–32]). In particular, the potential of *Mucor plumbeus* has been explored as a biocatalyst in synthetic chemistry [33–36], and in the depletion of PCP from culture media [28].

Abbreviations: PCP, pentachlorophenol; TeCHQ, tetrachlorohydroquinone; TCHQ, trichlorodihydroxybenzene (trichlorohydroquinone); MM, minimal media; GMM, minimal media containing glucose.

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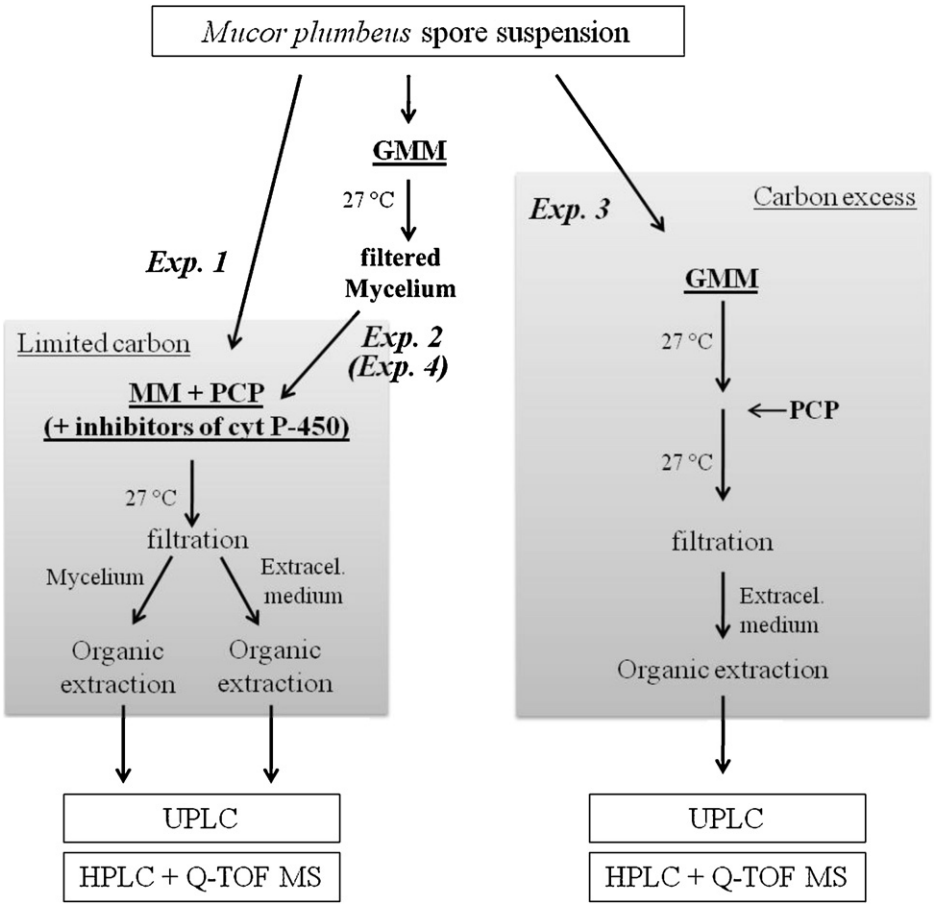

Fig. 1. Schematic view of the experimental approach.

Here a comprehensive study of the biotransformation of PCP by a strain of *M. plumbeus* is reported, testing the effect of different cultivation conditions (*viz.* type of inocula and availability of carbon). The PCP-derived metabolome was analysed by quadrupole time-of-flight (Q-TOF) high resolution mass spectrometry, leading to the identification of several PCP-derived metabolites. These included tetra- and tri-chlorohydroquinones and phase II-conjugated metabolites, namely sulfate, glucose, ribose and sulfate–glucose conjugates. The latter in conjugation with PCP or TeCHQ are here detected for the first time in fungi. Based on the data, a PCP transformation pathway for *M. plumbeus*, essentially through enzyme mediated intracellular steps, is proposed.

2. Materials and methods

2.1. Reagents

Pentachlorophenol, tetrachlorohydroquinone, 3,4-dimethoxybenzyl alcohol (veratryl alcohol, VA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), phenylmethanesulfonyl fluoride (PMSF), glycerol, D(+)-glucose, $K_2[HPO_4]$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, KCl, 1-aminobenzotriazole (1-ABT), 2-methyl-1,2-bis(3-pyridyl)-1-propanone (metyrapone) and Remazol Brilliant Blue R (RBBR) were obtained from Sigma–Aldrich (USA). $MgSO_4{\cdot}7H_2O$ and $NaNO_3$ were purchased from Fluka (Switzerland). Hydrogen peroxide 35 wt.% and sodium dodecyl sulfate (SDS) were obtained from Acros Organics (Belgium) and Dithiothreitol (DTT) from GE Healthcare (Sweden). All LC and MS solvents used were of the highest analytical grade.

2.2. Fungal strain and cultivation conditions

M. plumbeus Bonord (DSM 16513) belongs to the culture collection of Instituto de Biologia Experimental e Tecnológica (IBET, Portugal) [37,38]. The spore suspensions, prepared as previously described [28] and preserved in glycerol (10%, v/v) at −80 °C, were used to inoculate the liquid growth media (10^4 spores/mL). All submerged fungal cultures were incubated under controlled conditions, in the dark, at 27 °C and with orbital agitation (90 rpm).

The broth media (minimal media, MM) contained, *per* litre of water, 1 g K_2HPO_4; 3 g $NaNO_3$; 10 mg $ZnSO_4{\cdot}7H_2O$; 5 mg $CuSO_4{\cdot}5H_2O$; 0.5 g $MgSO_4{\cdot}7H_2O$; 10 mg $FeSO_4{\cdot}7H_2O$ and 0.5 g KCl. For some experiments, glucose was added to the culture media, to a final concentration of 10 g/L (glucose minimal media, GMM), unless otherwise stated. The pH of the media was adjusted to 6 with phosphoric acid. PCP was added to the autoclaved media, from a concentrated stock solution (28.2 mM) prepared in ethanol or dimethyl sulfoxide (DMSO). To prepare the RBBR solid media (0.02%, w/v), broth MM, containing 1 g/L of glucose, was converted to a gel with 1.5% (w/v) agar (Himedia, India).

2.3. PCP biotransformation

PCP biotransformation by *M. plumbeus* was monitored in MM or GMM, containing 15.0 μM or 18.8 μM of PCP, respectively. An overview of the experimental approach is depicted in Fig. 1. The MM containing PCP was inoculated with spores (Exp. 1) and with

mycelia (Exp. 2). The mycelia (16.0 ± 2.4 mg/mL, fresh weight) were recovered from a two-week-old culture in GMM using vacuum assisted filtration, with glass fibre prefilters (Millipore, USA) and washed with an excess of MM. When GMM was used, the addition of PCP to the media was done after three days of incubation (Exp. 3). Mycelia biomass in GMM at the time of PCP addition was 3.2 ± 0.9 mg/mL, fresh weight.

Cultures in PCP containing media (50 mL, triplicates) were incubated under controlled conditions (see above) for two months (Exp. 1) or for seven days (Exps. 2 and 3). An additional replicate of Exp. 2 was completed using ^{13}C labelled PCP. Abiotic controls (non-inoculated) and, when appropriate, negative controls (PCP free media), were also prepared for each set of experiments, all in triplicate. In order to analyse PCP degradation and glucose consumption (if applicable) over time, aliquots of the culture media were collected, diluted to half with methanol, and frozen at −20 °C, until analysis by chromatography.

At the end of the incubation time, both mycelia (recovered as described above) and filtered culture media were also collected, and preserved at −20 °C prior to extraction. The culture filtrates, acidified to pH 1–2 with phosphoric acid, were extracted twice with ethyl acetate (1:1, v/v). The combined organic extracts were dried under a soft nitrogen flow, the pellet equilibrated in a minimal volume of methanol (concentrated 100× (Exp. 1), 17× (Exps. 2 and 3) and 68× (Exp. 2 with ^{13}C labelled PCP) when compared to the culture media) and preserved at −20 °C, until analysis. The frozen mycelia were first macerated with a mortar and pestle, immediately homogenised in a solution of diluted phosphoric acid (pH 1–2) (6:1, v/w fresh mycelium), and processed as described above.

2.4. In vivo inhibition of cytochrome P-450

MM containing PCP and specific cytochrome P-450 inhibitors were inoculated with mycelia as described in Section 2.3 for Exp. 2 (Exp. 4). The inhibitors were 1-ABT (0.8 mM) and metyrapone (2.0 mM) which, when used alone or in combination, infer the potential involvement of cytochrome P-450 in the degradation of PCP. After incubation for 24 h, these cultures, and the corresponding negative controls (cultures in MM solely with PCP), were processed as described above (Fig. 1).

2.5. Enzymatic assays

Mycelia (recovered as described in Section 2.3) were used to inoculate GMM containing 18.8 μM of PCP (and 1 g/L glucose). After 20 h of incubation, PCP was virtually exhausted, and both mycelia and filtrate were processed as described in Section 2.3, with the exception that PMSF was added to the filtrates (to a final concentration of 1 mM) in order to inhibit protease activity. These were concentrated (*ca.* 50×) and equilibrated in tartrate buffer (0.1 M, pH 4.5), using an Amicon concentration device (Millipore, USA), with a cut-off of 10 kDa. Protein quantification was performed using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad, USA).

The contribution of extracellular lignin modifying enzymes in the degradation of PCP, if any, was evaluated by incubating fractions containing 20 μg/mL of protein, at 25 °C, in a tartrate buffer (0.1 M, pH 4.5) containing ABTS (1 mM) or VA (2 mM) without or with 0.1 mM H_2O_2, respectively. The corresponding oxidised products were quantified spectrophotometrically at 420 nm and 310 nm, respectively. Catalase activity in the protein extracts (0.1 M phosphate buffer, pH 7) was tested using the H_2O_2 *in vitro* decomposition method [39].

The contribution of extracellular enzymes in the degradation of PCP was also tested by incubating fractions containing 20 μg/mL of protein, in tartrate buffer (0.1 M, pH 4.5) containing PCP (67.6 μM). The residual PCP concentration, after 17 h of incubation, was measured by liquid chromatography (see Section 2.6.1). Positive controls using a commercial preparation of pure fungal enzymes, (laccase from *Trametes versicolor* and LiP, Sigma, USA), were conducted in parallel. The frozen mycelia were covered with tartrate buffer (0.1 M, pH 4.5) containing PMSF (1 mM) and allowed to thaw overnight at 4 °C. Fractions of the mycelia, before and after denaturation (incubation at 99 °C for 18 min, in tartrate buffer (0.1 M, pH 4.5) with 2% SDS (w/v) and 60 mM DTT), were placed onto the surface of RBBR solid media, and its decolouration monitored for 24 h, while incubated at 27 °C, in the dark.

2.6. PCP and PCP-derived metabolites analyses

2.6.1. Liquid chromatography

PCP and PCP-derived metabolites, either in aliquots of the culture media diluted to half with methanol, or in the organic extracts (see Section 2.3), were analysed by ultra-performance liquid chromatography (UPLC). When applicable, glucose was also quantified by high performance LC (HPLC), using a method previously described [28].

For the UPLC analysis a Waters Acquity^{TM} chromatograph, with a Photodiode Array Detector (PDA), temperature controlled autosampler, and column, was used (Waters Corporation, USA). Data acquisition was accomplished with the Empower 2 software, 2006 (Waters Corporation, USA). Injections of the extracts (3 μL) were made using a 10 μL loop, partial-loop mode, with needle overfill. The chromatographic separations were carried out using an Acquity UPLC HSS C18 column (2.1 × 150 mm, 1.8 μm particle size) (Waters Corporation, USA), set at 35 °C. The mobile phase, at a flow rate of 0.4 mL min^{-1}, consisted of a solution of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B), set as follows: a linear gradient of 10–95% B in 5.7 min, followed by 1.3 min to reach 100% B, 1.5 min to return to the initial conditions, and 1.5 min to re-equilibrate the column. The chromatographic profiles were acquired at 212 nm. Comparison with standards was used to identify PCP and TeCHQ, with retention times in min (t_R) of 5.9 and 3.9, respectively. PCP quantification limits were 0.1–15 mg/L.

2.6.2. Liquid chromatography–high resolution mass spectrometry (LC–HRMS) (Q-TOF)

The chromatographic separation (injection volume of 3 μL) was performed on an Agilent 1200 RRLC (Agilent, USA) using an Ascentis Express C18 (150 × 2.1 mm, 2.7 μm particle size) column from Supelco (USA), set at 50 °C. The mobile phase, at a flow rate of 300 μL min^{-1}, consisted of a solution of 0.1% formic acid (solvent A) and a solution of acetonitrile containing 0.1% formic acid (solvent B), set as follows: 10% B for 1 min, followed by a linear gradient of 10–95% B in 4.7 min, 1.3 min to reach 100% B, 3 min of 100% B, 0.5 min to return to the initial conditions, and 5.5 min to re-equilibrate the column. The HPLC system was coupled to a hybrid quadrupole time-of-flight QSTAR Elite (Applied Biosystems/MDS SCIEX, USA). The MS acquisition was performed in negative ionization operating in full scan (m/z 70–600) mode. Spray parameters were IS-4200, DP-60; FP-190; DP2–15; IRD 6; IRW 5; TEM 400 °C with N_2 as curtain (CUR = 50) and nebulizer (NEB = 50). The Q-TOF instrument was calibrated with taurocolic acid (1 pmol/μL) using the ions at m/z 79.9568 and 514.2844. MS data was processed using the Analyst QS 2.0 software. Compound identification was performed on the basis of the accurate mass of target analyte compared with those of elemental compositions provided by the software. For better identification, comparison of isotopic clusters was also performed. The presence of a certain metabolite was confirmed if errors lower than 5% between experimental and theoretical isotopic clusters were obtained.

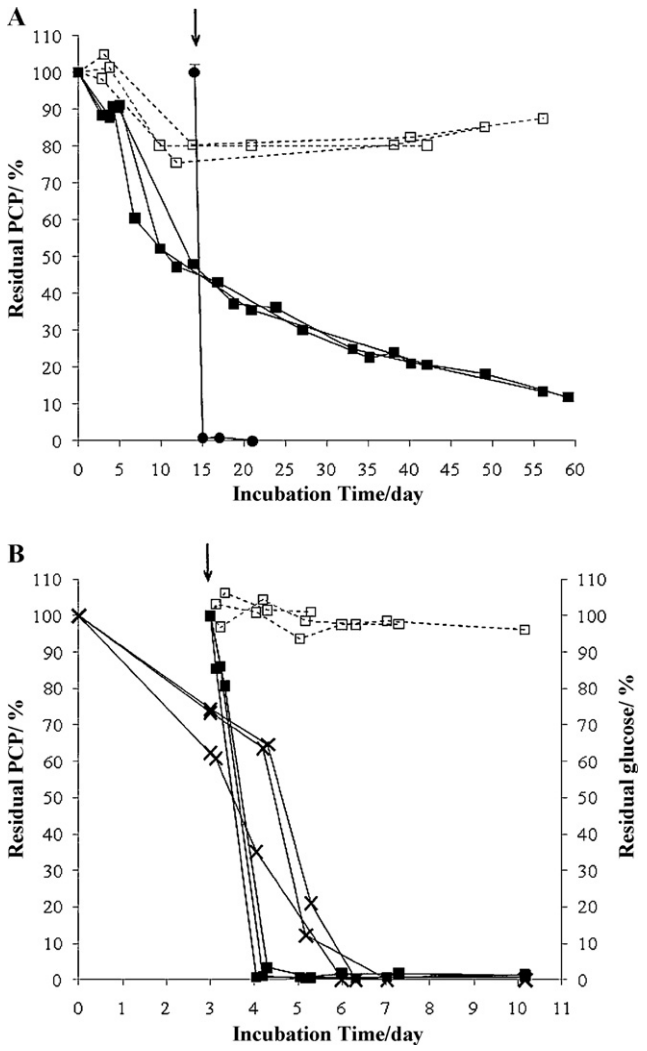

Fig. 2. Time dependence of PCP degradation in *Mucor plumbeus* cultures in media containing PCP, (A) without or (B) with glucose (MM and GMM, respectively). In the cultures grown from mycelia in GMM, the stage of PCP addition to the media is highlighted with an arrow. Full and open symbols indicate PCP residual concentration, expressed as a percentage, in the cultures and in abiotic controls, respectively. Residual glucose concentration (×), expressed as percentage, is also shown in (B).

3. Results

3.1. Time course of PCP degradation by M. plumbeus

PCP depletion from the culture media along the cultivation time was quantified by UPLC and is depicted in the graphs (Fig. 2). Each time point corresponds to the estimate of a replicate culture, except for Exp. 2 where the mean value of the three replicates is represented. When spores were used as inoculum (Exp. 1), after two months a very low amount of mycelium biomass was formed, <0.25 mg/mL, fresh weight. PCP depletion from the MM progressed slowly but efficiently. It reached 50% after fifteen days, and 91% at the end of the incubation (Fig. 2A). In contrast, when mycelia were used as inoculum, 24 h of incubation were sufficient for PCP to become virtually absent from both tested media, either MM (Exp. 2) or GMM (Exp. 3) (Fig. 2A and B, respectively). Total mycelial biomass (fresh weight) at the end of the incubation time was ~16 and ~6 mg/mL in MM and GMM, respectively. In the GMM, its depletion was complete before the glucose was exhausted (Fig. 2B). The abiotic degradation was estimated to be 5% for short (≤7 days) and 20% for long (>15 days) incubation times.

3.2. Chromatographic analysis of M. plumbeus extracts

The culture filtrates, at the end of the incubation time, were extracted and their chromatographic profiles resolved (see Fig. 3). When considering the differential chromatographic profile of cultures grown in PCP relative to controls, *i.e.* selecting only peaks absent from the media, solvent or control samples, five "new" peaks were detected. These peaks were similar regardless of the cultivation conditions used. With the exception of TeCHQ peak (t_R = 3.9), the remaining ones could not be preliminarily identified through comparison with standard compounds.

Chromatographic profile of mycelial fractions grown in MM is exemplified in Fig. 4 (Exp. 2). Only one differential, unidentified, peak was detected in comparison with that of a control culture (PCP free). Importantly, the amount of PCP extracted from mycelia, at the end of the incubation, was only 0.37% of the initial PCP added to the cultures.

3.3. Identification of PCP-derived metabolites by LC–HRMS (Q-TOF)

The presence of several PCP-derived metabolites in the culture filtrates (organic extracts) was confirmed by the LC–HRMS using a Q-TOF instrument obtaining accurate mass measurements with errors below 5 ppm. The PCP-derived metabolites, identified in extracts collected after growth from spores in MM with PCP (Exp. 1), are shown in Table 1. Tetrachlorohydroquinone (TeCHQ) and trichlorodihydroxybenzene (trichlorohydroquinone) (TCHQ), as well as several phase II conjugates (glucose–PCP, glucose–TeCHQ, ribose–TeCHQ, sulfate–TeCHQ and glucose–TCHQ), and diconjugates (sulfate–glucose–PCP and sulfate–glucose–TeCHQ) are included. The corresponding Total Ion Chromatogram (TIC) and, for peaks identified as TeCHQ (t_R = 7.70), sulfate–TeCHQ (t_R = 7.70) and sulfate–glucose–TeCHQ (t_R = 5.97), the extracted ion chromatograms and the resultant MS spectra, are also shown in

Table 1
Pentachlorophenol and PCP-derived metabolites in *Mucor plumbeus* cultures grown from spores in MM containing PCP, identified after two months, by LC–HRMS (Q-TOF).

Transformation product	Elemental composition[a]	Retention time (min)	Accurate *m/z*	Theoretical mass	Error (mDa)	Error (ppm)
Glucose–trichlorodihydroxybenzene conjugate	$C_{12}H_{12}Cl_3O_7$	3.69	372.9646	372.9654	0.8	2.1
Glucose–tetrachlorohydroquinone conjugate	$C_{12}H_{11}O_7Cl_4$	5.02	406.9274	406.9264	1.0	2.5
Ribose–tetrachlorohydroquinone conjugate	$C_{11}H_9Cl_4O_6$	5.52	376.9179	376.9169	0.7	1.9
Glucose–pentachlorophenol conjugate	$C_{12}H_{10}Cl_5O_6$	5.88	424.8945	424.8926	1.9	4.5
Sulfate–glucose–pentachlorophenol conjugate	$C_{12}H_{10}Cl_5O_9S$	5.88	504.8486	504.8493	0.7	1.4
Sulfate–glucose–tetrachlorohydroquinone conjugate	$C_{12}H_{11}Cl_4O_{10}S$	5.97	486.8848	486.8832	1.6	3.3
Trichlorodihydroxybenzene	$C_6H_2Cl_3O_2$	6.95	210.9134	210.9125	0.9	4.3
Tetrachlorohydroquinone	$C_6HCl_4O_2$	7.70	244.8740	244.8736	0.4	1.6
Sulfate–tetrachlorohydroquinone conjugate	$C_6HCl_4O_5S$	7.70	324.8285	324.8298	1.3	4.0
Pentachlorophenol	C_6Cl_5O	8.94	262.8401	262.8397	0.4	1.5

[a] Elemental composition of $[M-H]^-$ ions.

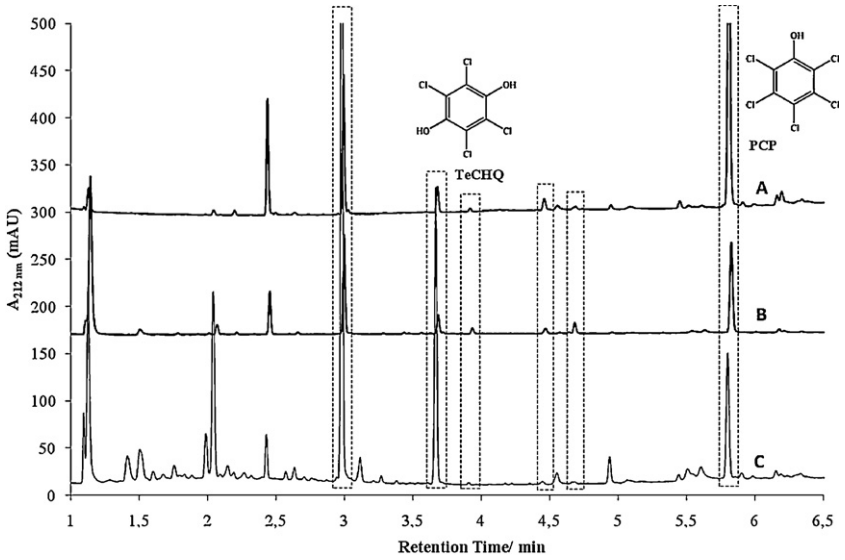

Fig. 3. UPLC chromatographic profiles of *Mucor plumbeus* culture filtrates (organic extracts), at the end of the incubation time, after growth in media containing PCP. The cultures were grown from (A) spores (Exp. 1) and (B) mycelia (Exp. 2) in MM, and from (C) mycelia in GMM (Exp. 3). The organic extracts were 5× concentrated, compared to the culture media. Peaks associated with PCP transformation are highlighted.

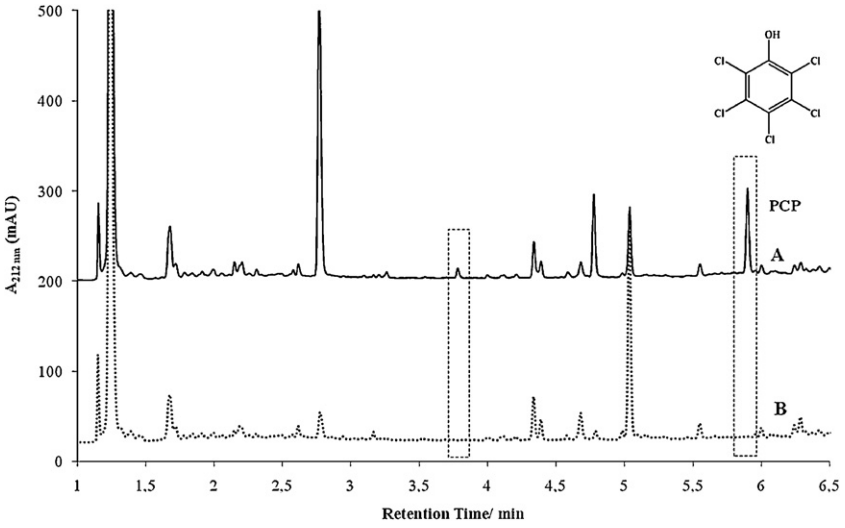

Fig. 4. UPLC chromatographic profiles of *Mucor plumbeus* mycelial fraction (organic extract), at the end of the incubation time. The cultures were grown in (A) MM with PCP and chromatogram B represents the negative control. Peaks associated with PCP transformation are highlighted.

Table 2
Pentachlorophenol-derived metabolites identified by LC–HRMS (Q-TOF) in the organic extracts of *Mucor plumbeus* culture filtrates (F) and mycelial fractions (M). The cultures were grown in media containing PCP: MM inoculated with spores or mycelia (Exp. 1 and Exp. 3, respectively); GMM inoculated with mycelia (Exp. 3) and in MM containing cytochrome P-450 inhibitors (1-ABT and/or metyrapone) inoculated with mycelia (Exp. 4). The list of metabolites identified in the abiotic controls is also shown.

Transformation product	Elemental composition[a]	Abiotic controls	MM Exp. 1	MM Exp. 2		GMM Exp. 3	MM Exp. 4
			F	F	M	F	F
Glucose–PCP conj.	$C_{12}H_{10}Cl_5O_6$	n.d.	√	√[c]	n.d.	n.d.	√
Sulfate–PCP conj.	$C_6Cl_5O_4S$	n.d.	n.d.	n.d.	√	n.d.	√
Sulfate–glucose–PCP conj.	$C_{12}H_{10}Cl_5O_9S$	n.d.	√	√	n.d.	√	√
TeCHQ	$C_6HCl_4O_2$	n.d.[b]	√	√	√	√	√
Glucose–TeCHQ conj.	$C_{12}H_{11}O_7Cl_4$	n.d.	√	√	√	√	√
Ribose–TeCHQ conj.	$C_{11}H_9Cl_4O_6$	n.d.	√	n.d.	n.d.	n.d.	n.d.
Sulfate–TeCHQ conj.	$C_6HCl_4O_5S$	n.d.	√	√	√	√	√
Sulfate–glucose–TeCHQ conj.	$C_{12}H_{11}Cl_4O_{10}S$	n.d.	√	n.d.	n.d.	n.d.	n.d.
TCHQ	$C_6H_2Cl_3O_2$	n.d.	√	√[c]	n.d.	n.d.	n.d.
Glucose–TCHQ conj.	$C_{12}H_{12}Cl_3O_7$	n.d.	√	√	n.d.	√	n.d.

√, detected; n.d., not detected.
[a] Elemental composition of $[M-H]^-$.
[b] Trace amounts detected in the GMM control.
[c] Detected in an experiment using ^{13}C-labelled PCP.

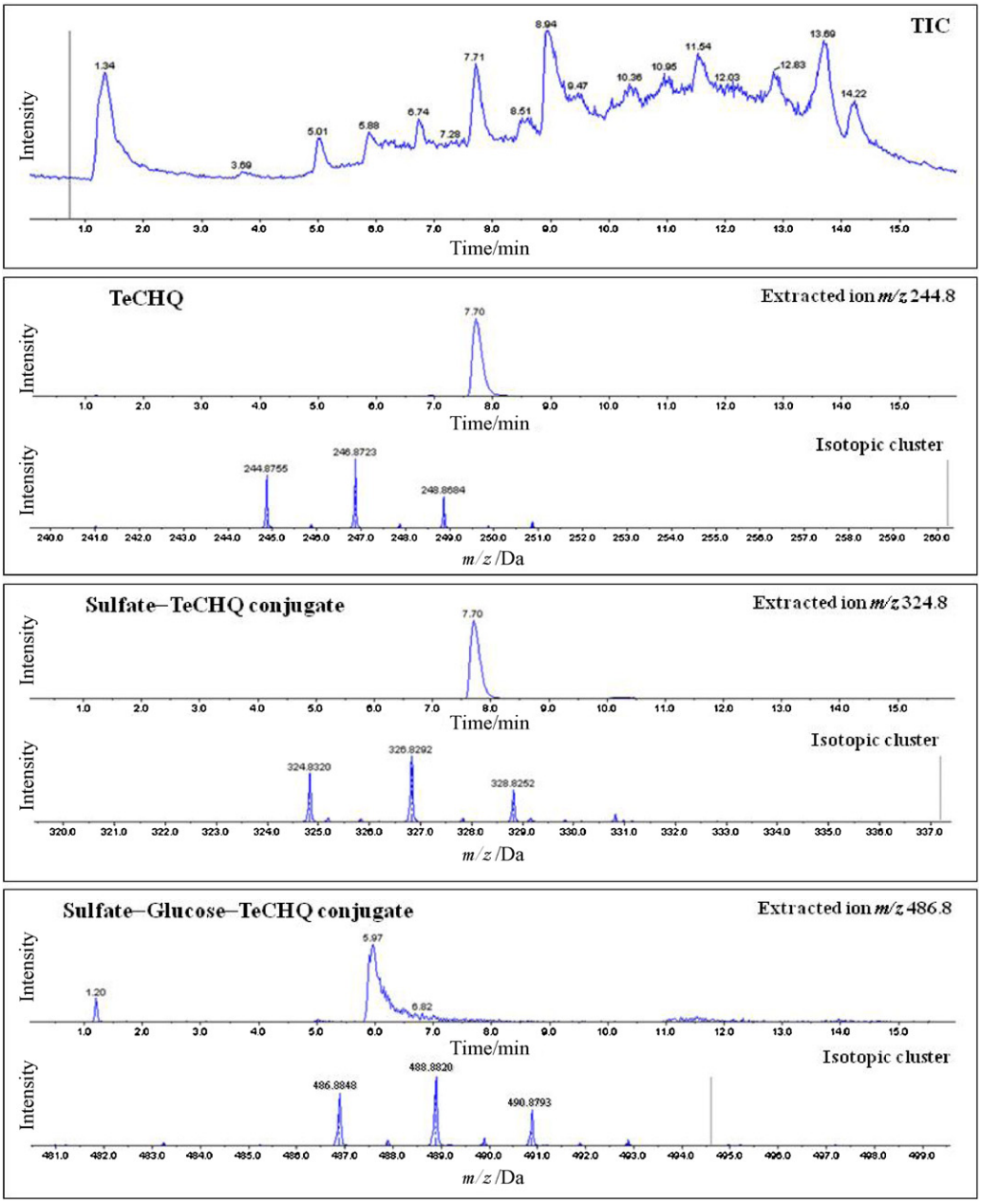

Fig. 5. Total Ion Chromatogram of an organic extract prepared from culture filtrates of *Mucor plumbeus* grown from spores in MM containing PCP. The extracted ion chromatogram and MS spectra for peaks corresponding to TeCHQ (t_R = 7.70), sulfate–TeCHQ conjugate (t_R = 7.70) and sulfate–glucose–TeCHQ conjugate (t_R = 5.97) are also shown.

Fig. 5. Table 2 integrates the PCP-derived metabolites identified in all filtrate cultures (organic extracts) (Exps. 1–4), and in a mycelial extract (Exp. 2). The latter only includes TeCHQ and some of its phase II-conjugates (glucose–TeCHQ and sulfate–TeCHQ). A trace amount of TeCHQ was identified in the GMM abiotic control.

3.4. *In vivo* inhibition of cytochrome P-450 during PCP degradation

The role played by the cytochrome P-450 of *M. plumbeus* in PCP transformation was evaluated *in vivo* in MM containing PCP. The enzyme inhibitors, namely 1-ABT and metyrapone, were added to the media immediately before its inoculation with mycelia (Exp. 4). PCP depletion from media after 24 h was >95% (Table 3), which was comparable to that of the negative controls, without enzyme inhibitors. Not surprisingly, both cultures, with or without enzyme inhibitors, reported an identical profile of PCP-derived metabolites (Table 2).

Table 3
Pentachlorophenol residual concentrations after *Mucor plumbeus* growth in MM containing PCP, in the presence or absence of cytochrome P-450 inhibitors. The inhibitors were 1-ABT and metyrapone, used alone or in combination.

Pentachlorophenol residual concentration (%)	
in vivo – 24 h incubation	
MM	99.0 ± 0.11
MM + 1-ABT	99.0 ± 0.07
MM + metyrapone	97.7 ± 0.60
MM + metyrapone + 1-ABT	95.5 ± 3.87

Table 4
Enzymatic assays *in vitro* with *Mucor plumbeus* mycelia and extracellular protein extracts, previously grown from mycelia in GMM containing PCP. Their ability to transform RBBR, ABTS, VA and PCP was determined. Catalase *in vitro* activity was also shown. Tests with pure commercial enzymes (positive control) and abiotic controls were also included.

Culture fraction	*Mucor plumbeus* grown in GMM containing PCP			
	RBBR decolouration	ABTS/VA oxidation	H_2O_2 decomposition	PCP residual concentration (%)
Mycelia	√	n.d.	√	n.d.
Mycelia after denaturation treatment	×	n.d.	n.d.	n.d.
Extracellular protein extract	×	×	√	97.9 ± 0.6
Abiotic control	×	×	×	102.2 ± 5.5
Positive control (commercial enzymes)	√	√	n.d.	63.3 ± 2.3

√, detected; ×, not detected; n.d., not determined.

3.5. Enzymatic assays

The evaluation of any contribution from extracellular lignin modifying enzymes in the degradation of PCP was also evaluated. The extracellular protein extracts, prepared from filtrate cultures grown for 20 h in GMM containing PCP, were unable to oxidise ABTS and VA (ligninolytic model substrates) (Table 4). These extracts showed catalase activity (Table 4). It has been reported that in the presence of oxidising enzymes the RBBR solid media becomes decolourised (colourless halo) [40]. Media decolouration after a short incubation period (6 h) was observed in the presence of mycelia (Table 4), but not in the corresponding negative control (*viz.* mycelia after denaturation treatment), nor in the extracellular protein extract (Table 4). The extracellular proteins were also unable to transform PCP *in vitro* as depicted in Table 4. Positive controls, either with pure commercial enzymes (Table 4) or with a protein extract from an Ascomycota strain displaying ligninolytic activity (data not shown) were prepared in parallel. These controls further confirm the absence of *M. plumbeus* extracellular ligninolytic enzyme activity under the conditions used here.

4. Discussion

Fungal biodegradation of PCP has been extensively researched and several strains, distributed across all phyla, have been recognised for this ability ([10] and references therein). Such capacity is often associated with the production of extracellular ligninolytic enzymes. This assumption focused previous research on Basidiomycota strains ([10,14,41] and associated references), neglecting the Zygomycota strains, in particular *Mucor* [22,24,26,28,42]. However, in the context of bioremediation the Mucorales show significant competitive advantages in contaminated soils [29,43], even with PCP [22]. Some stress-tolerant *Mucor* spp. can grow at high temperatures, with limited water and nutrients and in the absence of oxygen [44].

This study focused on the elucidation of the PCP degradation pathway by an environmental strain of *M. plumbeus*, previously observed to efficiently remove PCP from media, with or without glucose [28]. This strain was able to germinate from spores even in media with PCP concentrations >15 μM, an inhibiting concentration for *P. chrysosporium* [18]. The observed depletion of PCP in *M. plumbeus* cultures was efficient, but influenced by the total mycelial biomass (Fig. 2). While it progressed very slowly when cultures were grown from spores, it was virtually complete in the first 24 h when grown from mycelia. Accordingly, the amounts of mycelial biomass formed were <0.25 mg/mL and ≥3.2 mg/mL (fresh weight). The amount of PCP recovered from the mycelia, *i.e.* adsorbed onto the cell surface and bioaccumulated, was only 0.37% of the initial PCP and its abiotic degradation was <20%.

PCP-derived metabolites in *M. plumbeus* cultures, at the end of the incubation time, were identified by LC–HRMS (Q-TOF) (Tables 1 and 2). None of the PCP-derived metabolites were detected in the abiotic controls, with the exception of a trace amount of TeCHQ in GMM (Table 2). Its abiotic formation might have played a minor contribution to PCP depletion [45]. The PCP peak was evident in the organic extracts tested (Figs. 3 and 5), despite being virtually absent in the chromatograms of culture filtrates (data not shown, represented in Fig. 2). This is probably due to the low chemical stability of some phase II-conjugates in ethyl acetate [46,47].

The metabolites identified include TeCHQ (also detected in the UPLC chromatograms, Fig. 3) and TCHQ, as well as several phase II-conjugates or diconjugates (Tables 1 and 2). The diversity of metabolites detected in cultures grown from mycelia (Exp. 2), with the exception of ribose–TeCHQ and sulfate–glucose–TeCHQ, was similar to that grown from spores (Exp. 1) (Table 2). The diversity of metabolites was influenced by the mycelial biomass (Exp. 1 and Exp. 2), the incubation time (Exp. 2 and Exp. 4, seven days and 24 h, respectively) and the carbon availability (MM or GMM, Exp. 2 and Exp. 3, respectively). However, taken as a whole, the data suggest that the degradation pathways used by *M. plumbeus* in all the experimental conditions tested (Fig. 1), were indistinguishable.

All the conjugate groups detected, with the exception of the sulfate–glucose, have previously been observed in fungi, even if never detected in conjugation with PCP itself [46–50]. Some fungal strains, and in particular *Mucor* spp. [48,51], have been previously observed to form phase II-conjugates with xenobiotics [48–50]. Their formation reduces toxicity and increases water-solubility [50]. This generally involves the activity of enzymes that work in tandem. First, the xenobiotic is usually oxidised by oxidative enzymes, mainly, cytochrome P-450 monooxygenases; then, the oxidised compound is conjugated intracellularly with endogenous compounds (*e.g.* glutathione, sugar residues or sulfate) by a transferase [50]. The latter, in particular a glutathione S-transferase, has already been purified from *Mucor circinelloides* (previously *M. janvanicus*) [52].

PCP can also undergo direct phase II metabolism via conjugation to its hydroxyl group [53]. It appears that, glucose-PCP conjugates were rapidly formed in *M. plumbeus* cultures. Their formation even in cultures grown in the absence of glucose (Exp. 1) might involve the mobilisation of the fungal spores' reservoirs, such as trehalose [54]. Due to the use of an ethanolic PCP stock solution, the initial ethanol concentration in the cultivation media was 9.1 mM. Therefore one cannot exclude the involvement of gluconeogenesis, as suggested previously [55]. The formation of glucose conjugates by *Penicillium canescens* during exposure to dibenzofuran was also independent of the carbon source used for the cultivation of the fungus [46].

Sulfate–glucose diconjugates (*i.e.* PCP and TeCHQ), identified here for the first time in fungi, have been detected, *e.g.* in aquatic crustaceans exposed to pyrene [56]. The addition of the sulfate negative charge to the glucose conjugate might stimulate its excretion [56]. The sulfate–glucose–PCP was detected in *M. plumbeus* filtrates,

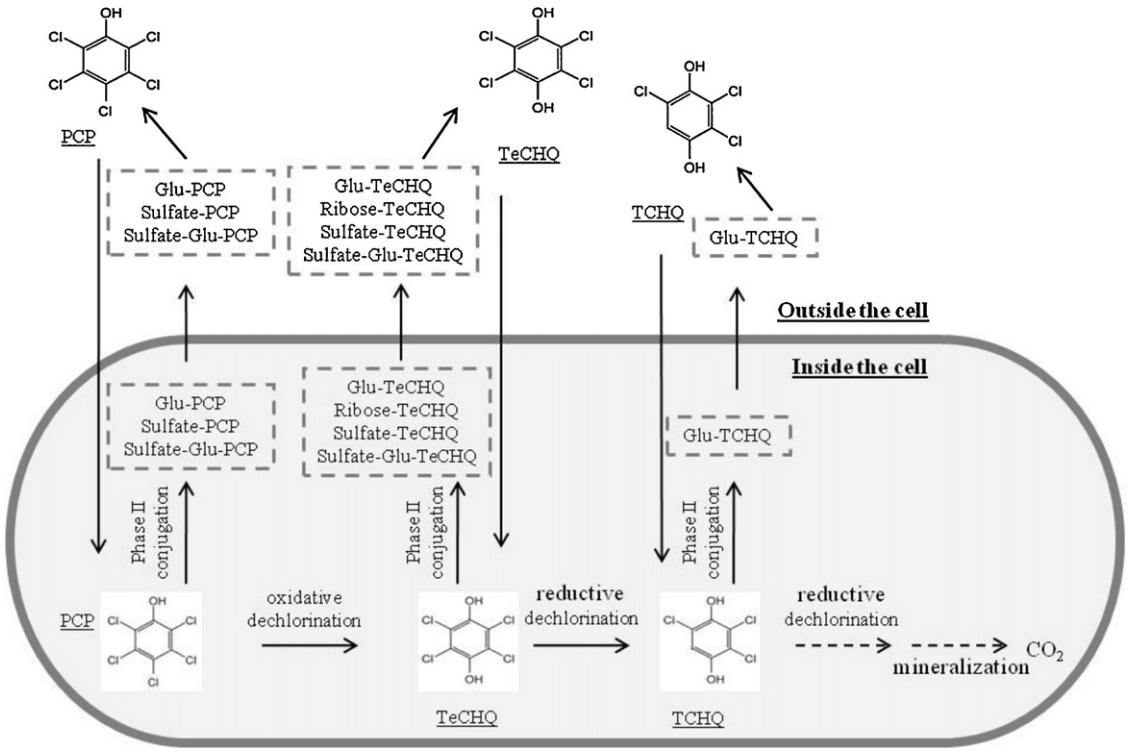

Fig. 6. Schematic view of the PCP degradation pathway in *Mucor plumbeus* cultures, proposed to explain observations from this study.

regardless of the mycelial biomass, while the precursor conjugate, glucose–PCP, was not detected in cultures of higher mycelial biomass (Exp. 3, GMM). Even though PCP is considered a poor substrate for sulfation [57], sulfate–PCP (previously reported in aquatic organisms [53]) was detected in this study. It was observed in *M. plumbeus* cultures grown from mycelia, namely in the filtrate (Exp. 4, 24 h) and in the mycelial fraction (Exp. 2, seven days). The absence of sulfate–PCP from the remaining filtrates suggests its short half-life in the extracellular media.

M. plumbeus cultures also formed ribose conjugates, namely ribose–TeCHQ. This type of conjugate has been observed before in other fungi, usually in very low amounts when compared with the glucose equivalents [48,58]. Their late-accumulation in a mineral growth media inoculated with *Paecilomyces lilacinus* mycelia was observed [58]. Therefore, and not surprisingly, in this study, ribose–TeCHQ conjugates were only detected in the filtrate of Exp. 1 where the longest incubation period was used.

Cytochrome P-450 apparently does not play a major role in PCP transformation by *M. plumbeus*. *In vivo*, regardless of the presence or absence of specific enzyme inhibitors, PCP was efficiently depleted from the media (Table 3) and the same PCP-derived metabolites were formed (Table 2, Exp. 4). The oxidative dechlorination of PCP to TeCHQ in *Mucor ramosissimus* was suggested by Szewczyk et al. to involve cytochrome P-450 activity [24], but used different experimental conditions, *e.g.* different culture media and concentrations of PCP. PCP depletion from media in this study was complete in less than 24 h, strongly suggesting that *M. plumbeus* uses an alternative pathway to mediate PCP oxidation.

Mucor species are generally devoid of ligninolytic activity, despite recent reports of ligninolytic enzymes of a *M. racemosus* strain [59]. Extracellular protein extracts of *M. plumbeus* were unable to oxidise, under the conditions tested, ABTS, VA and RBBR (Table 4). *M. plumbeus* cultures were also grown in media containing VA, which might further stimulate the production of these enzymes [60]. Though VA was depleted to half after three weeks of growth, the resulting extracellular protein extracts failed to oxidise PCP (data not shown). Other extracellular enzymes might be involved in PCP degradation, as suggested for a tyrosinase from *Amylomyces rouxii* [42]. However, the tested extracellular protein extracts were unable to transform PCP, apparently ruling out the involvement of any other extracellular enzymes (Table 4). A soluble fraction extracted from the mycelia was also unable to oxidise RBBR, while the corresponding mycelial debris rapidly decolourised the media (data not shown). This, along with the demonstration that PCP and RBBR are both rapidly transformed by *M. plumbeus* mycelia, strongly suggests that the oxidation of PCP to TeCHQ is mediated by an intracellular enzyme, most probably located in the membrane.

The identification of TCHQ and glucose–TCHQ conjugates in *M. plumbeus* cultures highlights the involvement of reductive dechlorination of TeCHQ during PCP degradation. In *P. chrysosporium* this step is mediated by a membrane-bound glutathione S-transferase and a soluble glutathione conjugate reductase [61,62]. In the present study, glutathione-conjugates were not detected. Even though metabolites with fewer chlorine atoms than TCHQ were not detected, one cannot rule out the hypothesis that subsequent dechlorination might occur in *M. plumbeus* cultures. This hypothesis needs further investigation.

Based on these observations, a pathway for PCP transformation by *M. plumbeus*, which begins with PCP intracellular mobilisation, is proposed (Fig. 6). The data suggest that PCP oxidation to TeCHQ, its subsequent reductive dechlorination to TCHQ, and the formation of phase II-conjugates, evolves exclusively in the mycelial fraction. The conjugates can be mobilised to the extracellular matrix, where deconjugation takes place, allowing the detection of TeCHQ and TCHQ metabolites. The deconjugation reaction might involve catalase activity [50], which was also detected in the extracellular protein extracts (Table 4). Despite numerous studies on PCP degradation pathway in fungi, this is the first time that a pathway which combines a set of conjugation and oxidative-reductive dechlorination reactions is described.

5. Conclusion

The potential of *M. plumbeus* for PCP bioremediation is highlighted in this study. Efficient transformation, even in low carbon availability conditions, is most likely to involve intracellular PCP oxidation to TeCHQ, its subsequent reductive dechlorination to TCHQ and formation of several phase II-conjugates. The latter include some sulfate–glucose conjugates identified here for the first time in fungi. *M. plumbeus* is able to rapidly transform PCP to less toxic compounds. It uses a series of oxidative–reductive dechlorination reactions, while it takes advantage of phase II conjugation reactions to keep the intracellular levels of the toxic compounds low. This capacity of *M. plumbeus* suggests an important role to protect less tolerant strains in PCP-contaminated environments.

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