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Oxidoreductases provide a more generic response to metallic stressors than hydrolases in soil fungi: new ecotoxicological insights

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Abstract

The present study investigates the effect of metals on the secretion of enzymes from 12 fungal strains maintained in liquid cultures. Hydrolases (acid phosphatase, β -glucosidase, β -galactosidase and N-acetyl- β -glucosaminidase) and ligninolytic oxidoreductases (laccase, Mn- and lignin-peroxidases) activities, as well as biomass production were measured in culture fluids from fungi exposed to Cu or Cd. Our results showed that all fungi secreted most of the selected hydrolases and that about 50 % of them produced a partial oxidative system in the absence of metals. Then, exposure of fungi to metals led to the decrease of biomass production. At the enzymatic level, Cu and Cd modified the secretion profiles of soil fungi. The response of hydrolases to metals was contrasted and complex, and depended on metal, enzyme and fungal strain considered. By contrast, the metals always stimulated the activity of ligninolytic oxidoreductases in fungal strains. In some of them, oxidoreductases were specifically produced following metal exposure. Fungal oxidoreductases provide a more generic response than hydrolases, providing a physiological basis for their use as biomarkers of metal exposure in soils.

Keywords

Hydrolases, Ligninolytic oxidoreductases, Metals, Biomarkers, Secretion profiles, Ecotoxicology

1. Introduction

Soils are often intensively exploited to meet human needs and agricultural practises (application of fertilizers, pesticides, sewage sludge...), resulting in their contamination by organic chemicals or metals. Because of their non-degradability and persistence in soils, metals may alter soil biological activity and microbial community structure and functioning (Akmal et al. 2005; Heijerick et al. 2006; Nziguheba and Smolders 2008). Soil enzymatic activities are usually used as global endpoints to assess human impacts on biochemical processes driven by microorganisms (Gianfreda et al. 2005; Riah-Anglet et al. 2015). This assumes a consortium response of microbial taxa to chemical stressors. However, the effects of contaminants on soil enzymes are still controversial in the literature because of their likely functional redundancy within microbial species and abiotic factors influencing their expression levels (Lebrun et al. 2012; Riah et al. 2014). In addition, the functional potential of soil microorganisms and changes in the expression of enzymatic systems in response to a metallic stress are poorly understood, limiting hence the predictive interest of enzymatic tools for environmental risk assessment.

Fungi constitute a major and ubiquitous biomass in most terrestrial ecosystems. By their ability to secrete numerous enzymes, they are involved in initial stages of natural polymer decomposition, nutrient cycling and energy fluxes (Nannipieri et al. 2003; Baldrian 2006). White-rot basidiomycetes have been extensively studied because they secrete oxidoreductases, i.e. laccases and peroxidases, able to degrade not only the recalcitrant polymer lignin but also aromatic contaminants (Novotný et al. 2009; Uhnáková et al. 2009). These fungi also secrete hydrolases involved in the mineralization of chitin or cellulose, as described in *Pleurotus ostreatus* or *Trametes versicolor* (Baldrian et al. 2005; Lebrun et al. 2010). With respect to fungi living in soils, some ascomycetes and zygomycetes have been reported to efficiently degrade lignino-cellulosic materials (Borgia and Mehnert 1982; Kovacs et al. 2009). Despite their significance for environmental processes, information on functional diversity for soil fungi remains far less extensive than for white-rot fungi.

During experiments in liquid cultures, we have previously demonstrated that Cu, Cd, Zn and Pb alone or in cocktail alter the secretome of *T. versicolor*, decrease the diversity of secreted proteins and lead to posttranslational modifications (Lebrun et al. 2011a). At the enzymatic level, these metal exposures modulate the expression of both hydrolases and oxidoreductases, as also observed in *P. ostreatus* (Baldrian et al. 2005). We have thus suggested that the secretion patterns of fungi could be tools for metal ecotoxicity assessment. However, the responsiveness of fungi to metals may vary one species to another, which is still unknown. Thus, establishing generic responses within a soil community despite individual sensibilities is a crucial step to prove the relevance of using fungal enzymes as biomarkers of metallic stress.

This study aims at determining whether the functional changes in soil fungi can be used as indicators of metal ecotoxicity in soils. We used ten fungi of different phyla isolated from agricultural soils as well as two efficient lignin-degraders, *T. versicolor* and *Irpex lacteus* (Novotný et al. 2009; Uhnáková et al. 2009). To assess their functional potential, fungi were screened for their ability to secrete two classes of enzymes in liquid cultures, i.e. hydrolases commonly monitored in soils (acid phosphatase, β -glucosidase, β -galactosidase and N-acetyl- β -glucosaminidase) and ligninolytic oxidoreductases rather specific to fungal communities (laccase, Mn- and lignin-peroxidases), when exposed or not to Cu or Cd.

2. Materials and Methods

2.1. Cultures of soil fungi and metal exposures

Ten fungal strains came from the Microorganisms of Agro-Environmental Interest (MIAE) collection of UMR Agroécologie (INRA Dijon, France). Fungal strains frequently isolated from cropped soils were chosen among different phyla, i.e. 2 Basidiomycetes: *Thanatephorus cucumeris* (MIAE 08.006) and *Stereum rugosum* (MIAE 08.005); 5 Ascomycetes: *Fusarium solani* (MIAE Fspi1), *Fusarium oxysporum* (MIAE Fo47), *Penicillium brevicompactum* (MIAE 08.003), *Trichoderma atroviride* (MIAE Tri10) and *Verticillium albo-atrum* (MIAE Vert1); 3 Zygomycetes: *Mucor hiemalis* (MIAE 08.009), *Mucor racemosus* (MIAE 08.00X) and *Mortierella alpina* (MIAE 08.007). We also used two white-rot fungi, *Trametes versicolor* (ATCC 32745) and *Irpex lacteus* (Fr. 617/93), known for their ligninolytic activity. The later strain was obtained from the Culture Collection of Basidiomycetes (CCBAS, Institute of Microbiology, Prague, Czech Republic).

All soil fungi were successfully grown into a modified Kirk medium containing 10 g/L maltose, 2.5 g/L yeast extract, 2 g/L KH_2PO_4 , 0.5 g/L MgSO_4 , 0.1 g/L CaCl_2 and mineral salts (Cu, Zn, Fe and Mn at 0.1 mg/L for each metal) (Lebrun et al. 2011b). A mycelium mat on agar plugs (10 mm diam.) was inoculated into 10 mL of liquid medium in 150 mL Erlenmeyer flasks. For metal exposures, 100 μL of CuSO_4 or CdSO_4 sterilized by filtration (0.2 μm pore size) were added into the liquid cultures at final concentrations of 0.1, 1 or 3 mM. Controls were performed without metal addition. Fungal cultures were carried out statically in the dark at 25 °C. After 7 d-exposure, mycelia were harvested by a nylon screen (40 μm) and dried for 48 h at 80 °C to determine the fungal biomass. The mycelium free cultures were filtrated (0.2 μm -pore size) and used for enzymatic activity assays. For each fungal strain, three independent cultures were performed per treatment (i.e. 252 fungal cultures).

2.2. Enzymatic activity assays

Acid phosphatase (EC 3.1.3.2), β -glucosidase (EC 3.2.1.21), β -galactosidase (EC 3.2.1.23) and N-acetyl- β -glucosaminidase (EC 3.2.1.30) activities were measured using their respective

substrates of conjugated p-nitrophenyl (Sigma-Aldrich). Assays were carried out in 96-well microplates by mixing 160 μ L of substrate solution (25 mM) in citrate/phosphate buffer (pH 4.5, 0.1 M) with 40 μ L of enzymatic samples, followed by incubation at 37 °C. After 45 min, 50 μ L of Na₂CO₃ (1 M) was added to stop the reaction. The liberation of p-nitrophenol by enzymatic hydrolysis was determined at 405 nm. The standard solutions of p-nitrophenol were treated in the same way as samples (Lebrun et al. 2010).

Laccase (EC 1.10.3.2), manganese- and lignin-peroxidases (EC 1.11.1.13 and EC 1.11.1.14) activities were respectively monitored by the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid at 420 nm, 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one at 334 nm in the presence of MnSO₄ and H₂O₂ and veratrylic alcohol at 310 nm in the presence of H₂O₂, as previously described (Lebrun et al. 2011b).

Activities measured in triplicate were expressed in U/g dry weight (d.w.) of fungal biomass. One unit of activity was defined as the amount of enzyme that catalyzed 1 μ mol of substrate in 1 min. Controls performed from boiled enzymatic extracts to inactivate the enzymes, were treated in the same way as the samples. Significant differences in enzymatic levels between controls and exposed fungi were determined by a Mann–Whitney test ($P < 0.05$).

3. Results and Discussion

3.1. Functional diversity in soil fungi

The ability of soil fungi to produce both extracellular hydrolases and oxidoreductases in liquid cultures is presented in Figure 1. No production of tested enzymatic activities was detected in *M. alpina*. With respect to hydrolases, they were widely detected in soil fungi. Except *M. hiemalis* found to secrete only β -glucosidase, all other fungi secreted acid phosphatase, β -glucosidase, β -galactosidase and N-acetyl- β -glucosaminidase in various amounts according to the strain. The most efficient producers of extracellular hydrolases were *S. rugosum*, *P. brevicompactum* and *T. atroviride*. With respect to oxidoreductases, their secretion was partial in soil fungi. Laccase activity was detected in three fungal strains (*T. versicolor*, *T. cucumeris* and *T. atroviride*), Mn-peroxidase activity in two strains (*T. versicolor* and *I. lacteus*) and lignin-peroxidase activity in one strain (*F. oxysporum*). *T. versicolor* was the most efficient producer of extracellular laccases and Mn-peroxidases with 36.7 and 2.9 U/g d.w. respectively.

Since soil enzymes reflect the structure and functions of microbial communities, various hydrolases are commonly measured to assess the biological activity and quality of soils (Nannipieri et al. 2003; Lebrun et al. 2012). However, soil enzymes inquire on global biochemical processes without giving information on microbial species actively involved in these functions. To date, links between the microbial diversity and expression of its functions is still poorly documented (Riah-

Anglet et al. 2015). Here we show that a panel of soil fungi of different phyla isolated from agricultural soils secrete various hydrolases involved in the cycling of phosphor and the degradation of natural polymers such as cellulose and chitin. Therefore, our study supports the functional redundancy of hydrolases within fungal community, suggesting that diverse species are able to perform the same environmental functions in terrestrial ecosystems.

The oxidoreductases involved in the degradation of lignin, a recalcitrant polymer in the environment, are known to be rather specific to fungal communities, especially saprophytic basidiomycetes such as *T. versicolor* and *I. lacteus* (Dhouib et al. 2005; Baldrian 2006). Here we show that some soil fungi displayed an oxidative ligninolytic system, i.e. the basidiomycete *T. cucumeris* and the ascomycetes *T. atroviride* and *F.oxysporium*. Although these fungi are not described as efficient lignin-degraders, their laccase activity has been reported in other studies (Crowe and Olsson 2001; Verdin et al. 2004; Dhouib et al. 2005). Even if the secretion of oxidoreductases is not prevalent in soil fungi unlike hydrolases, our study provides an insight of the diversity of enzymes secreted by soil fungi and subsequently, contributes to improve knowledge on their implication in environmental processes.

3.2. Fungal growth as affected by metals

The ability of soil fungi to grow in the presence of Cu or Cd was presented in Table 1. Overall, metal exposures led to a decrease in fungal biomass production with a dose-dependent effect higher for Cd than for Cu. For Cu at 1 and 3 mM, the biomass production decreased of 87 and 54 % as averages for all tested fungi. For Cd, the biomass production decreased from the concentration of 0.1 mM in most soil fungi and downed to 26 % for the highest concentration of 3 mM. This is consistent with the fact that Cd is reported as being more toxic for white-rot fungi than Cu, which is an essential element toxic in excess (Baldrian, 2003). Even if fungi display defense mechanisms to prevent metal toxicity (synthesis of metallothioneins, metal-complexing organic acids...), they have an energy cost which is at the expense of growth (Lebrun et al. 2010). Our results support that metal contamination of terrestrial environments cause changes in structure and composition of fungal communities.

3.3. Responses of extracellular hydrolases to metals

Since the growths were strongly limited during metal exposures at 3 mM (Table 1), only enzymatic secretion patterns of soil fungi exposed for 7 d to Cu or Cd at 0.1 and 1 mM are presented in Figure 2. The response of hydrolases to metallic stress varied one fungal species to another, being sometimes stimulated or inhibited by Cu or Cd. Total inhibitions of hydrolase activities occurred out mostly for the exposure to Cd at 1 mM, i.e. the acid phosphatase in *M. racemosus* and *T. cucumeris*, β -galactosidase in *T. versicolor* and N-acetyl- β -glucosaminidase in *M. racemosus*. In *M. hiemalis*, Cu

and Cd specifically induced the secretion of β -galactosidase, not detected in unexposed cultures.

It has been reported that hydrolase activities tend to be inhibited by different metals, essential or not, during liquid cultures of the white-rot fungi *T. versicolor* or *P. ostreatus* (Baldrian et al. 2005; Lebrun et al. 2010). Here, we have not established generic responses of hydrolases to metals in soil fungi, highlighting the risk of extrapolating data obtained from the individual to the scale of community. The hydrolase activities tested in our study are commonly used to assess human impacts on terrestrial ecosystems. High levels of their activities are often assumed to reflect a high soil quality, whereas low levels mean toxic effects of contaminants on biological systems (Gianfreda et al. 2005; Lebrun et al. 2012). However, our results suggest that hydrolases are not relevant descriptors of metal contamination, at least for the fungal community, because of the great intracommunity variability of their response to metallic stress. This assumption is consistent with the absence of significant impacts of Cu on hydrolase activities in real field situations (Lebrun et al. 2012).

3.4. Responses of extracellular oxidoreductases to metals

The secretion of oxidoreductases was generally stimulated by Cu and Cd in fungal strains displaying a ligninolytic system (Fig. 2), until 95-fold for the laccase of *T. atroviride* exposed to Cu at 1 mM as the greatest example. Besides, Cu induced specific productions of laccase in *M. racemosus*, *F. oxysporum*, *S. rugosum* and *F. solani*, Mn-peroxidase in *T. atroviride* and lignin-peroxidase in *T. versicolor*. A specific production of Mn-peroxidase was also detected in *V. albo-atrum* exposed to Cd at 1 mM. A single inhibitory effect was observed for the lignin-peroxidase of *F. oxysporum* exposed to Cd at 1 mM.

It is known that Cu and Cd stimulate the secretion of ligninolytic oxidoreductases as reported in the basidiomycetes, *T. cucumeris* and *T. versicolor* (Crowe and Olsson 2001; Lebrun et al. 2011a). However, we show that these activities are also stimulated by metals in soil ascomycetes (i.e. *T. atroviride* and *F. oxysporum*). The specific production of lignin-peroxidase in the presence of Cu has previously been described in *T. versicolor* (Lebrun et al. 2010). Here we demonstrate that such specific productions of oxidoreductases in response to metallic stress occur in 50% of tested fungal strains. Both the genericity of response and induction of production of oxidoreductases can be explained by the presence of metal-responsive elements (MREs) on genes encoding for these fungal enzymes, involving in their transcription. Such MREs have been characterized on genes coding for laccase, Mn- and lignin peroxidases in *P. ostreatus* and *T. versicolor* (Johansson and Nyman 1996; Giardina et al. 1999). Therefore, fungal oxidoreductases appears to be relevant tools for metal ecotoxicity assessment in soils. But this implies the development of reliable methods for the quantification of oxidoreductase activity levels in soil samples (Floch et al. 2007).

Conclusions

Our study demonstrates that soil fungi of different phyla are able to secrete both hydrolases and oxidoreductases involved in various environmental processes. The exposure of fungi to metals modifies their enzymatic secretion patterns. Despite of individual sensitivities, it is possible to establish generic responses within this soil community. Unlike the great variability of the response of hydrolases, the fungal oxidoreductases are either stimulated or specifically produced during metal stresses. Because of the genericity of their response, the fungal oxidoreductases could be relevant tools for metal ecotoxicity assessment in soils. Further studies will be performed in order to propose reliable methods for the quantification of their expression levels from soil samples.

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Table 1. Effect of Cu or Cd on biomass production in liquid cultures of soil fungi

Fungal strain	Control Dry weight (mg)	Biomass production (% of unexposed controls)					
		Exposure to Cu			Exposure to Cd		
		0.1 mM	1.0 mM	3.0 mM	0.1 mM	1.0 mM	3.0 mM
<i>T. versicolor</i>	51.1 \pm 1.5	96.8	78.5*	65.2*	96.4	43.1*	2.7*
<i>I. lacteus</i>	56.7 \pm 0.9	88.2*	35.5*	4.1*	75.2*	27.2*	3.2*
<i>T. cucumeris</i>	77.3 \pm 1.9	100.0	64.2*	36.5*	11.6*	3.6*	2.4*
<i>S. rugosum</i>	24.6 \pm 1.7	90.5	68.3*	31.2*	106.6	64.2*	25.2*
<i>F. solani</i>	37.8 \pm 1.0	140.0*	106.6	12.6*	100.5	69.2*	29.9*
<i>F. oxysporum</i>	37.5 \pm 1.8	122.1*	131.6*	49.3*	97.2	88.0	73.0*
<i>P. brevicompactum</i>	45.8 \pm 1.1	96.3	85.7*	78.3*	85.2*	35.8*	10.3*
<i>T. atroviride</i>	25.6 \pm 3.2	90.0	99.1	113.0	149.8*	92.6	10.6*
<i>V. albo-atrum</i>	44.7 \pm 1.4	103.0	117.7*	107.2*	91.1*	118.1*	64.1*
<i>M. hiemalis</i>	45.9 \pm 0.6	90.9*	87.0*	38.9*	89.6*	51.7*	41.1*
<i>M. racemosus</i>	58.1 \pm 1.7	101.7	97.7	74.9*	91.8*	67.4*	25.5*
<i>M. alpina</i>	47.1 \pm 2.3	96.7	68.7*	32.9*	80.4*	25.0*	26.7*
Average effect of metal (affected fungi)		101.4 (4/12)	86.7 (9/12)	53.7 (11/12)	89.6 (8/12)	57.2 (10/12)	26.2 (12/12)

* significant effect of metal compared to the unexposed control (U-test; $p < 0.05$)

Figure 1. Extracellular hydrolases and oxidoreductases secreted by soil fungi cultured for 7 days in liquid media. The hydrolases (PAC, acid phosphatase; GLU, β -glucosidase; GAL, β -galactosidase; NAG, N-acetyl- β -glucosaminidase) are represented by blue shades and the oxidoreductases (Mn-P, Mn-peroxidase; Li-P, lignin-peroxidase; LAC, laccase) are represented by red shades.

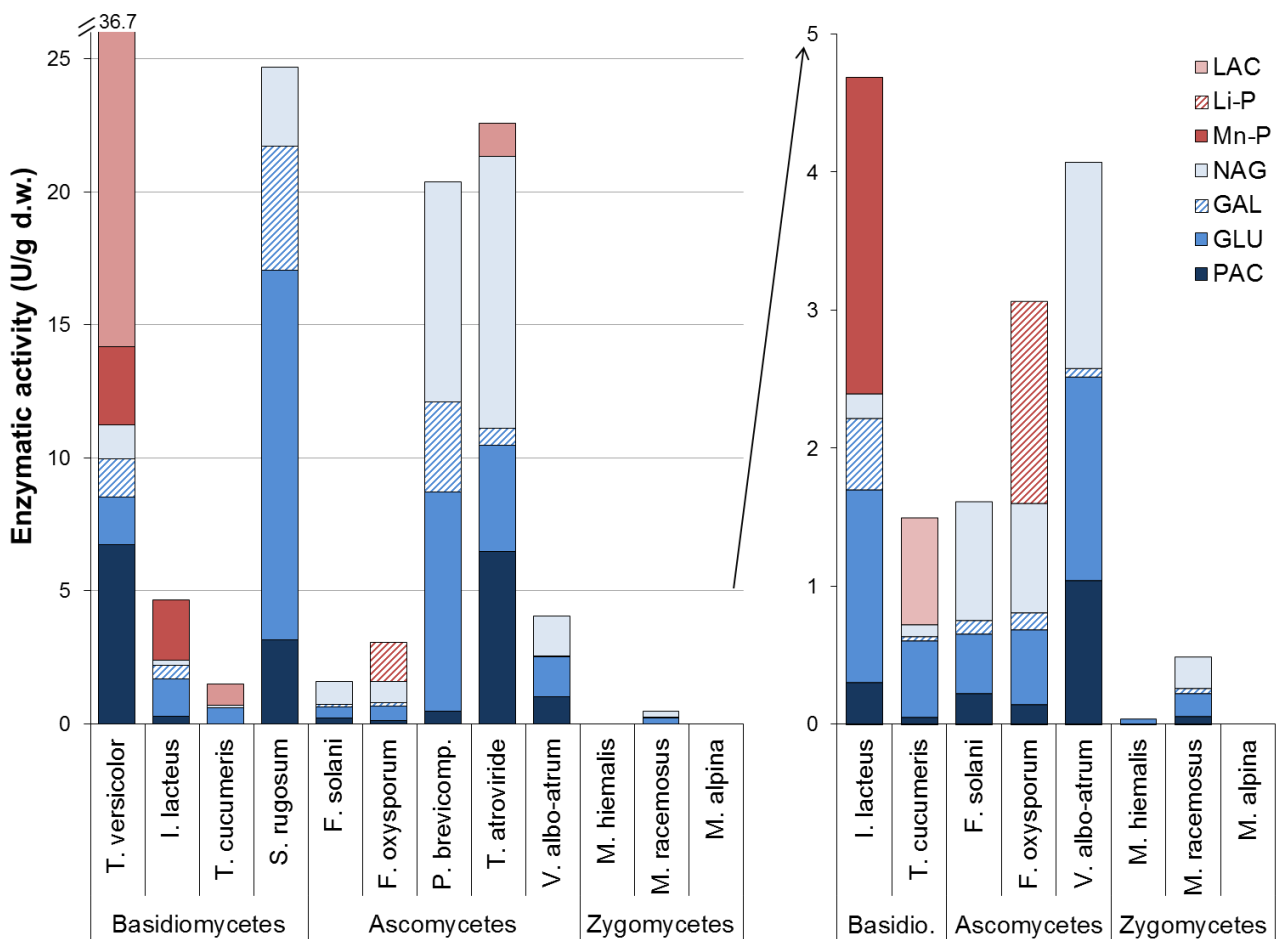


Figure 2. Effects of Cu or Cd on the production of extracellular hydrolases or oxidoreductases in soil fungi. Each symbol represents one fungal strain and the dotted line represents their relative basal production of enzymes in the absence of metals. When the symbol is on this dotted line, this means that the metal has not significant effect on the production of the considered enzyme compared to unexposed controls (U-test; $P < 0.05$). *S.P.* means a specific production of the enzyme in the presence of metal whereas no production is detected in unexposed controls. *T.I.* means a total inhibition of enzymatic activity in the presence of metal whereas it is detected in unexposed controls. Abbreviations: PAC, acid phosphatase; GLU, β -glucosidase and GAL, β -galactosidase for the hydrolases (blue background) and, LAC, laccase; MnP, Mn-oxidase and LiP, lignin-oxidase for the oxidoreductases (red background).

