Insights into the development of fungal biomarkers for metal ecotoxicty assessment: Case of Trametes versicolor exposed to copper

Jérémie D. Lebrun, Isabelle Trinsoutrot-Gattin, Karine Laval, Christian Mougin

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 29, No. 4, pp. 902–908, 2010

Printed in the USA
DOI: 10.1002/etc.101

INSIGHTS INTO THE DEVELOPMENT OF FUNGAL BIOMARKERS FOR METAL ECOTOXICITY ASSESSMENT: CASE OF *TRAMETES VERSICOLOR* EXPOSED TO COPPER

JÉRÉMIE D. LEBRUN,*†‡ ISABELLE TRINSOUTROT-GATTIN,‡ KARINE LAVAL,‡ and CHRISTIAN MOUGIN†

†INRA, UMR 251 Physico-chimie et Ecotoxicologie des SolS d'Agrosystèmes Contaminés, RD 10, 78026 Versailles, France

‡ESITPA, Equipe BioSol, 3 rue du Tronquet, 76134 Mont-Saint-Aignan, France

(*Submitted* 6 *May* 2009; *Returned for Revision* 31 *August* 2009; *Accepted* 23 *October* 2009)

Abstract—The relationship between the physiological state of fungi and the response of their functional system to metals is not known, limiting the use of fungal enzymes as tools for assessing metal ecotoxicity in terrestrial ecosystems. The present study attempts to establish how the development phases modulate the secretion of enzymes in the filamentous fungus *Trametes versicolor* after exposure to Cu. For that purpose, extracellular hydrolases (acid and alkaline phosphatases, aryl-sulfatase, β-glucosidase, β-galactosidase, and N-acetyl-β-glucosaminidase) and oxidoreductases (laccase, manganese and lignin peroxidases) were monitored in liquid cultures for 2 weeks. Copper was added during either the growth or the stationary phases at 20 or 200 ppm. Results of the present study showed that Cu at the highest concentration modifies the secretion of enzymes, regardless of the development phase to which the fungus was exposed. However, the sensitivity of enzyme responses to Cu depended on the phase development and the type of secreted enzyme. In a general way, the production of hydrolases was decreased by Cu, whereas that of oxidoreductases was highly increased. Furthermore, lignin peroxidase was not detected in control cultures and was specifically produced in the presence of Cu. In conclusion, fungal oxidoreductases may be enzymatic biomarkers of copper exposure for ecotoxicity assessment. Environ. Toxicol. Chem. 2010;29:902–908. © 2009 SETAC

Keywords—Filamentous fungi Hydrolases Laccase Mn-peroxidase Lignin peroxidase

INTRODUCTION

Soil pollution resulting from human activities is a worldwide problem with serious consequences for the health of the ecosystems. The release of metallic pollutants into the soil and the nondegradability of these elements have led to important accumulation in biota and often cause adverse effects on biological systems. The assessment of these effects, at the least on soil functioning, remains a research subject because of the lack of efficient tools. Soil microorganisms produce extracellular hydrolases that take part in the initial step of the mineralization of biopolymers such as cellulose or chitin and are also involved in the cycling of P, S, or N. As a consequence, these biochemical actors play an important role in soil functioning [1]. Thus, global hydrolytic activities are commonly measured in soils for many decades to assess their health, functioning, and quality in the presence of pollutants [2–4]. It is assumed that high levels of activity reflect high soil quality, whereas low levels mean toxic effects. Nevertheless, the physiological bases of these assumptions remain to be determined in filamentous fungi, which represent one of the largest biomasses in terrestrial ecosystems [5].

Saprophytic fungi, considered the predominant degraders of lignin, have been particularly studied for their ligninolytic oxidoreductases, such as laccases or peroxidases [6–8]. Environmental interest in this fungal set of enzymes has increased because of their ability to degrade a variety of organic xenobiotics [9–11]. Although fungi have mechanisms for metal tolerance [12], the activity of their extracellular enzymes has been shown to be modulated during the metal exposures in liquid cultures. Copper increases the extracellular laccase in different filamentous fungi [13–15]. It is also known that Cu induces the laccase transcription in *Trametes versicolor* [16]. Heavy metals, such as Cu, Zn, Mn, and Pb, modify the extracellular activity of cellulolytic and ligninolytic enzymes in *Pleurotus ostreatus* [15]. In this fungus, the cellulotytic activities were generally decreased, and the laccase activity was increased with all tested metals. Because of these functional modulations, it is assumed that fungal enzymes can be used as tools for assessing the metal ecotoxicity in soil.

Soil is a dynamic system in which fungi are in the growth or stationary phase. As a consequence, the fungal response to metals can depend on their development phase under environmental conditions. Because the relationship between the physiological state of fungi and the response of their enzymatic system to metal stress is not known, the predictive interest in fungal biomarkers to metal exposure is hence limited. Furthermore, the fungal responses to metals can also be modulated by other factors, such as the physicochemical characteristics of soil or the availability of metal. By an inoculation strategy, the influence of these factors on fungal enzymatic activities can be assessed in terrestrial systems. For example, the fungal biomass and laccase activity were monitored after an inoculation of *T. versicolor* or *Cunninghamella elegans* in soil contaminated by polycyclic aromatic hydrocarbons [17]. Thus, it is feasible to use fungi and their enzymatic system in bioassay studies. However, before performing studies taking into account the complex properties of soil, it is first necessary to improve our

* To whom correspondence may be addressed (jlebrun@versailles.inra.fr).

Published online 23 December 2009 in Wiley InterScience (www.interscience.wiley.com).

knowledge of the response of the enzymatic system of fungi to metal stress at the organism level in order to determine the relevance of using fungal enzymes as biomarkers of metal exposure.

The present study aims to establish how the development phases influence the response of *T. versicolor* to Cu at the functional level. Copper has been retained because it is a common contaminant of soils from many agrosystems, amounting to 100 to 1,000 ppm (mg/kg of soil), whereas geochemical values in noncontaminated soils are between 5 and 30 ppm [18–20]. Two main families of enzymes involved in terrestrial ecosystem functioning were monitored: hydrolases (β-glucosidase, N-acetyl-β-glucosaminidase, β-galactosidase, acid and alkaline phosphatases, and aryl-sulfatase) and oxidoreductases (laccase, manganese and lignin peroxidases) in liquid cultures of *T. versicolor*.

MATERIALS AND METHODS

Culture conditions and metal exposure

Trametes versicolor ATCC 32745 was grown in a liquid culture medium, containing maltose and ammonium tartarate as carbon and nitrogen sources [21]. A mycelium mat on agar plugs (10 mm diameter) was inoculated into 10 ml culture medium in 150-ml Erlenmeyer flasks. Cultures were carried out statically in the dark at 25°C. After 3 or 9 d of incubation, 100 μl of $SO_4Cu \cdot 5H_2O$ (VWR; >99% purity) sterilized by filtration (0.2-μm-pore-size membrane) were added to the liquid cultures at final concentrations of 20 or 200 ppm (mg Cu/L culture medium). Controls were realized without metal addition. The experiments were carried out with three independent replicates (three fungal cultures) per treatment (five conditions) and per sampling day (eight dates).

Sampling

At different days of incubation over a 16-d period, mycelia were harvested with a nylon screen (40 μm) and dried for 48 h at 80°C to monitor the fungal growth. This determination of the dry weights of fungal biomass was also used as an indicator of metal toxicity. The mycelium-free culture was used for the extracellular activities assays, quantification of total proteins by the Bradford method using bovine albumin as a standard [22], and total sugar amounts by the Nelson-Somogyi method [23].

For intracellular activities, mycelia were washed five times with ice-cold physiological water (NaCl, 9 g/L), frozen at −20°C for 24 h, and thawed to rupture cells. Mycelia were ground in a mortar to liberate intracellular enzymes. Membrane debris was separated from the ground extract by centrifugation at 6,000 *g* for 30 min at 4°C. Obtained supernatants were filtered through 0.45-μm-pore-size membranes and used to measure intracellular activities. For membrane activities, membrane debris pellets were washed twice with cold citrate/phosphate buffer (0.05 M, pH 5), centrifuged, and resuspended to measure membrane activities [24].

Activity assays

Hydrolase activities were assayed using their substrates of conjugated *para*-nitrophenyl (Sigma-Aldrich). Assays were carried out in 96-well microplates, by mixing 120 μl substrate solution (25 mM) in acetate buffer (0.1 M) with 80 μl enzymatic samples, followed by incubation at 37°C. After 45 min, 50 μl Na_2CO_3 (1 M) was added to stop the reaction. The liberation of *para*-nitrophenol by enzymatic hydrolysis of the substrate was determined at 405 nm. 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) were tested at pH 4.5; aryl-sulfatase (EC 3.1.6.1) at pH 7; and alkaline phosphatase (EC 3.1.3.1) at pH 10 [25,26]. The standard solutions of *para*-nitrophenol were treated in the same way as the samples.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic) acid (1 mM; $\xi_{420} = 36{,}000\ M^{-1}\ cm^{-1}$) at 420 nm in citrate/phosphate buffer (CPB; 0.1 M, pH 3.0) at 30°C [27]. Lignin and manganese peroxidases (EC 1.11.1.13 and EC 1.11.1.14, respectively) activities were monitored, respectively, by the oxidation of veratrylic alcohol (3 mM; $\xi_{310} = 9{,}300\ M^{-1}\ cm^{-1}$) at 310 nm in CPB (pH 3.0) in the presence of H_2O_2 (0.6 mM) [28] and the oxidation of 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one (1 mM; $\xi_{334} = 18{,}300\ M^{-1}\ cm^{-1}$) at 334 nm in CPB (pH 5.0) in the presence of $MnSO_4$ (0.1 mM) and H_2O_2 (0.4 mM) [29]. Solutions of enzymes were added to a final volume of 1 ml, and the kinetics were monitored for 30 s.

Assay mixtures containing membrane debris samples were centrifuged (10,000 *g* for 1 min) before absorbance reading. Intracellular, extracellular, and membrane activities were measured in triplicate and expressed in units per gram dry weight of fungal biomass (U/g dry wt). Controls were done with enzymatic samples boiled to inactivate the activities and were treated in the same way during the enzymatic assays. One unit of activity was defined as the amount of enzyme that catalyzed 1 μmol substrate in 1 min.

Statistical analyses

Mean and SEM were calculated from three independent sets for each biological variable measured. Statistical analyses were performed by XLStat, and $p < 0.05$ was taken to indicate significant differences.

RESULTS

Fungal development and metal toxicity

The growth of *T. versicolor* was first determined in order to identify the development phases over a 16-d period (Fig. 1). In the absence of metal, *T. versicolor* grew during 8 d. This growth phase was related to the consumption of available sugars in the liquid medium. The fungus then entered in stationary phase from 8 to 9 d when the source of carbon (sugars) was exhausted. This stationary phase was followed by a decline phase from 9 to 16 d as a result of cell lysis.

Copper was then added at 20 or 200 ppm at either day 3 or 9 of fungus incubation, during the growth or stationary phases, respectively. Whatever the concentration of Cu added during the growth phase (3 d), the metal had no effect on the growth of *T. versicolor* compared with the unexposed control (Fig. 1a and b). However, the fungus reached the stationary phase earlier for the concentration of 200 ppm, linked to a faster loss of sugars in the liquid medium (Fig. 1b). When Cu at 200 ppm was added in

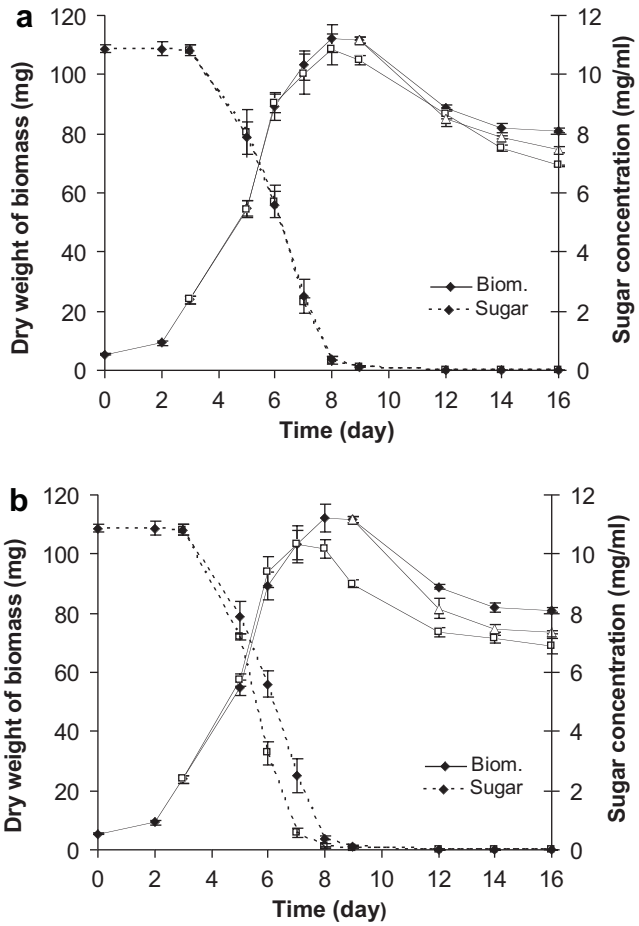

Fig. 1. Sugar concentration in culture medium and growth of *Trametes versicolor* exposed to Cu at 20 (**a**) or 200 (**b**) ppm. Unexposed (lozenges) or exposed to Cu added during either the growth (squares) or the stationary (triangles) phase. The values are means ± SE ($n = 3$); biom. = biomass.

the stationary phase (9 d), the lysis rate was significantly increased compared with the control (Fig. 1b).

Distribution of enzymatic activities

Hydrolase and oxidase activities were measured in extracellular, intracellular, and membrane compartments in the absence of metal to establish the localization of enzymes produced by *T. versicolor* during the growth and decline phases (Fig. 2). Aryl-sulfatase and lignin peroxidase activities were not detected regardless of the development phases. Among the detected activities, it could be observed that all hydrolase activities were principally associated with cells (>85% of total activities) in the growth phase (Fig. 2a). Acid phosphatase, β-glucosidase, β-galactosidase, and N-acetyl-β-glucosaminidase activities were distributed between intracellular and membrane compartments, except for alkaline phosphatase, which was only intracellular. By contrast, oxidase (laccase and Mn-peroxidase) activities were preferentially found in the extracellular compartment (>45% of total activities). This distribution was slightly affected in the decline phase (Fig. 2b). The principal difference appears for β-galactosidase activity, whose extracellular part was increased five times compared with the growth phase.

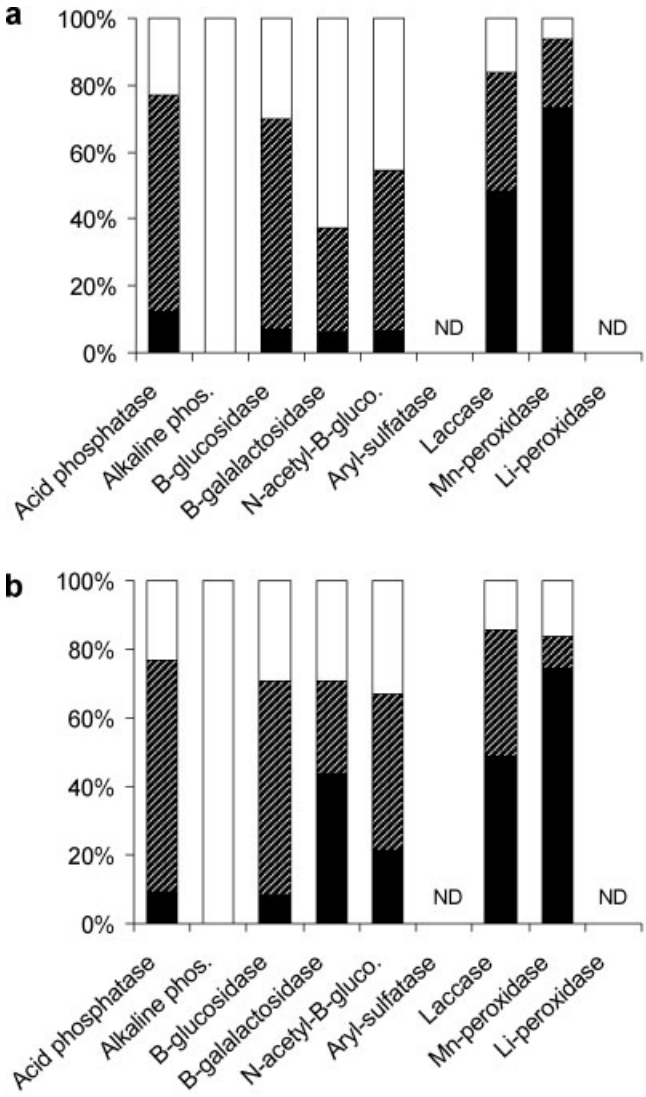

Fig. 2. Distribution of enzymatic activities in the different compartments of *Trametes versicolor* after 6 or 12 d of incubation, in growth (**a**) or stationary (**b**) phases, respectively. Extracellular (solid columns), membrane (hatched columns), and intracellular (open columns) compartments. The activities expressed in U/g dry weight were reported as percentages ($n = 9$). ND = not detected.

Protein secretion during fungal development

The regulation of protein secretion during the development of *T. versicolor* is presented in Figure 3. In the absence of metal, a first peak of secretion appeared during the growth phase, reaching 16.9 mg proteins/g dry weight of fungal biomass on day 3. A second peak occurred during the decline phase of the fungus, when the value of total secreted proteins amounted to 27.4 mg/g dry weight on day 14. The Cu addition at 20 ppm either in the growth or in the stationary phase slightly modified the protein secretion compared with the unexposed control (Fig. 3a). However, the metal at 200 ppm altered the regulation of secretion (Fig. 3b). Its addition during the growth phase led to a long-term effect by decreasing the stimulation of protein secretion occurring the decline phase. Despite variations in the amount of secreted proteins, Cu addition in the stationary phase did not prevent this stimulation.

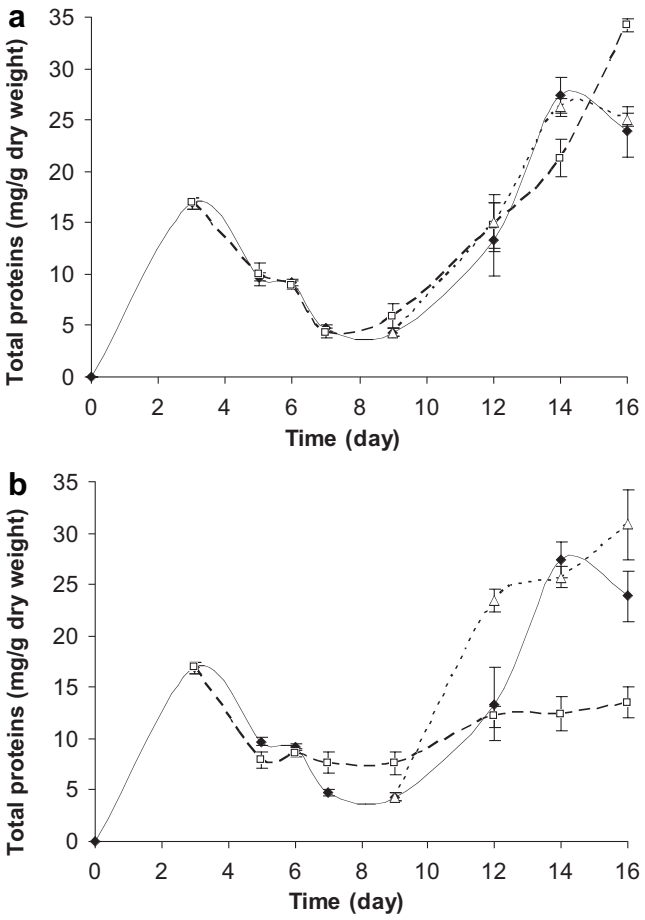

Fig. 3. Effect of copper at 20 (**a**) or 200 (**b**) ppm on the protein secretion by *Trametes versicolor*. Unexposed (lozenges) or exposed to Cu added either during the growth (squares) or stationary (triangles) phase. The values are means ± SE ($n = 9$).

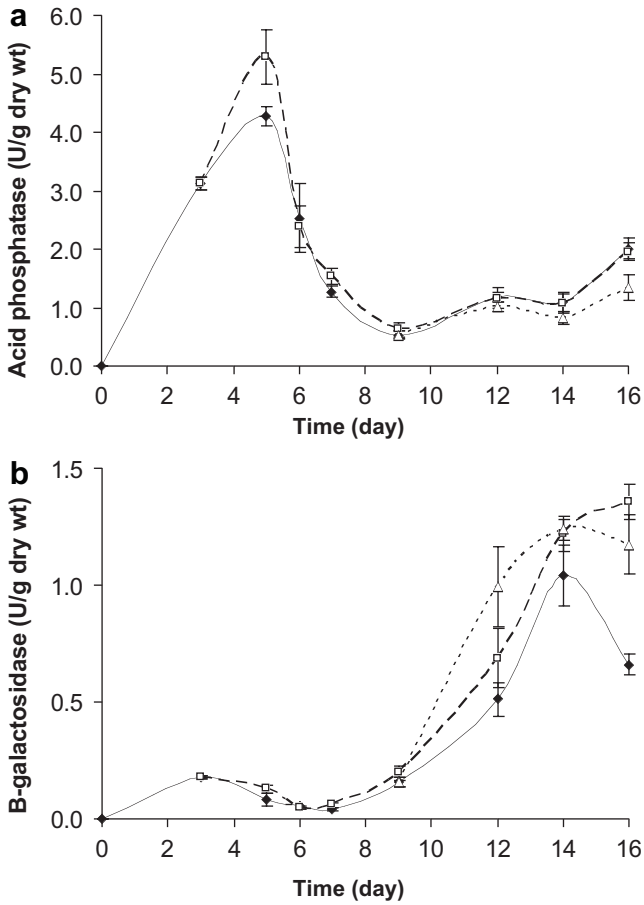

Fig. 4. Responses of extracellular acid phosphatase (**a**) and β-galactosidase (**b**) activities to Cu at 20 ppm in *Trametes versicolor*. Unexposed (lozenges) or exposure to Cu added during the growth (squares) or stationary (triangles) phase. The values are means ± SE ($n = 9$).

Secretion of hydrolases and oxidoreductases

Among extracellular enzymes detected and secreted in significant amounts by *T. versicolor* in the absence of Cu, two sets of enzymes can be distinguished according to their secretion profile under culture conditions. Each set had a peak of activity during either the growth or the decline phase related to the peaks of protein secretion of the fungus (Fig. 3 vs. Figs. 4 and 5). A first set, including acid phosphatase, β-glucosidase, and N-acetyl-β-glucosaminidase, was secreted mainly during the growth phase (Figs. 4 and 5). Peaks of these activities were at least twofold higher than their activity average over the culture period (Table 1). By contrast, a second set including β-galactosidase and oxidoreductases (laccase and Mn-peroxidase) was poorly produced during the growth phase and secreted mainly during the decline phase (Figs. 4 and 5). Peaks of β-galactosidase, Mn-peroxidase, and laccase activities were at least 2.5-fold higher than their activity average over the culture period (Table 1).

Effect of Cu on extracellular enzymes

The exposure of *T. versicolor* to Cu showed that the metal modifies the production of extracellular enzymes. However, this response is dose dependent. Applied at 20 ppm, Cu slightly altered the secretion profiles of hydrolases and oxidoreductases compared with the controls, regardless of the developmental phase at which the fungus was exposed (see examples in Fig. 4). Only the laccase production was significantly stimulated by a factor of 1.7 during the exposure period when Cu was added in growth phase (Table 1). Applied at 200 ppm, Cu highly modulated the extracellular activities (Fig. 5). In every exposure phase, except for β-glucosidase, all hydrolase activities were decreased by Cu (see N-acetyl-β-glucosaminidase, Fig. 5a). The β-glucosidase activity increased when the metal was added in the stationary phase (Fig. 5b). The production of extracellular oxidoreductases, such as laccase and Mn-peroxidase, was increased substantially by Cu at 200 ppm (see laccase, Fig. 5c). Moreover, the exposure of *T. versicolor* to Cu resulted in a specific response of lignin peroxidase produced only during the decline phase (Fig. 5d). However, the intensity of enzyme responses to Cu depended on the phase of development (Table 1). Acid phosphatase, N-acetyl-β-glucosaminidase, β-galactosidase, and peroxidases were more sensitive to a metal addition during the growth phase than during the stationary phase. This was a situation opposite to that for laccase.

DISCUSSION

Among filamentous fungi, the rot-white species has been studied especially for their ligninolytic oxidases. However,

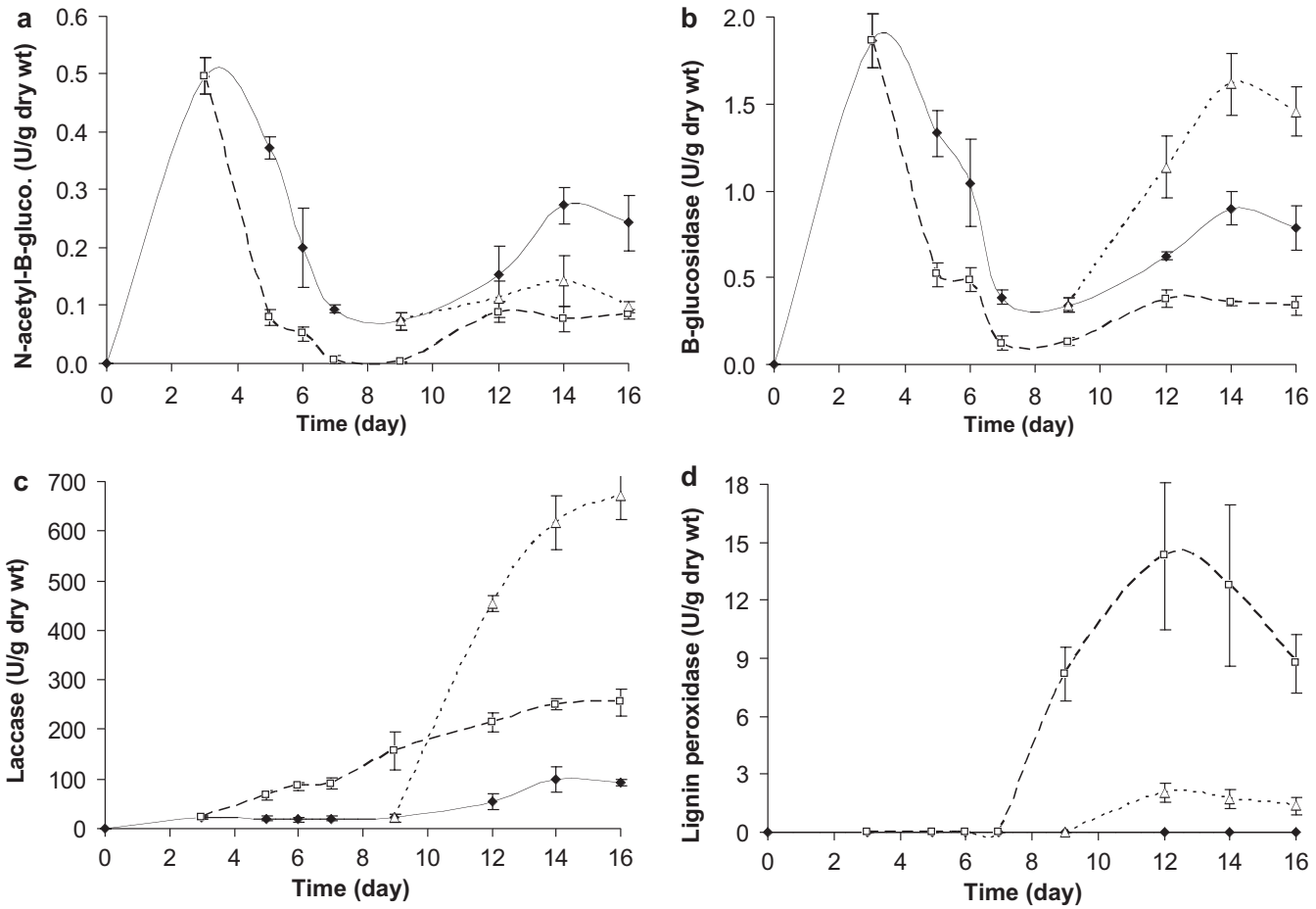

Fig. 5. Responses of extracellular hydrolase (**a**, N-acetyl-β-glucosaminidase; **b**, β-glucosidase) and oxidase (**c**, laccase; **d**, lignin peroxidase) activities to Cu at 200 ppm in *Trametes versicolor*. Unexposed (lozenges) or exposed to Cu added during the growth (squares) or stationary (triangles) phase. The values are means ± SE ($n = 9$).

knowledge of both their extracellular hydrolases and functional diversity is still limited. In the present study, it was shown that *T. versicolor* produced extracellular hydrolases, such as acid phosphatase or glycosidases (β-glucosidase, β-galactosidase and N-acetyl-β-glucosaminidase). These hydrolases are involved in the intake of phosphorus and the degradation of natural polymers such as cellulose or chitin. The distribution of enzymatic activities in different fungal compartments provided a snapshot of the localization of the enzymes. Hydrolases were essentially associated with cells, i.e., in membrane and intracellular compartments. This could be explained by the involvement of glycosidases in the degradation of disaccharides that can enter the cell by permeases [24]. By contrast, lignolytic enzymes known to depolymerase extracellular substrates [8,12] were preferentially found in the outside environment. This is in agreement with the identification of signal peptides involved in the secretory pathways in genes coding for lignin and Mn-peroxidases in *T. versicolor* [30].

The present study shows that the secretion of both total proteins and enzymes depends on the physiological state of

Table 1. Averages and peaks of extracellular activities (U/g dry wt) and effect of copper in cultures of *Trametes versicolor*

	Control		Copper addition			
			Growth phase		Stationary phase	
	Average	Peak (day)[a]	20 ppm	200 ppm	20 ppm	200 ppm
Acid phosphatase	2.00	4.26 (5)	107	52*	76	44
β-Glucosidase	0.93	1.86 (3)	142	43*	174	182*
N-acetyl-β-glucosaminidase	0.24	0.50 (3)	98	25*	89	55*
β-Galactosidase	0.29	1.04 (14)	138	21*	164	90
Mn-peroxidase	11.66	34.36 (14)	125	330*	76	149
Laccase	36.33	98.13 (14)	169*	418*	133	730*

[a] Number in parentheses indicates the day of activity peak. Effect of copper on activities is expressed as percentage of control.
* Significant effect of copper during the exposure period ($p < 0.05$).

T. versicolor under culture conditions. During the growth phase, there was a panel of oversecreted proteins, including acid phosphatase, β-glucosidase, and N-acetyl-β-glucosaminidase. During the decline phase, the protein secretion was highly stimulated, related to the increase of some activities, such as laccase, Mn-peroxidase, and β-galactosidase. When the fungus was subjected to nutrient exhaustion such as carbon limitation, it could thus activate metabolic alternative pathways associated with a new enzymatic production pattern. For example, it is known that the production of laccases and peroxidases is increased when medium carbon is exhausted [7]. A release of intracellular proteins resulting from the cell lysis observed during the decline phase cannot be excluded. However, ligninolytic oxidoreductases are known to be constitutively produced at the extracellular level in several fungi of different taxons [6,7,12,13]. Moreover, β-galactosidase activity is basically extracellular in *Hypocrea jecorina,* also known for several filamentous fungi, including *Aspergillus* and *Penicillium* species [24]. In addition, alkaline phosphatase, which was only intracellular, was not found in the extracellular medium during cell lysis.

With their adaptation mechanisms to different stressors, including metal tolerance, and their involvement in nutrient cycles [1,5,12], fungi are of ecological relevance. In the present study, Cu, one main metallic contaminant in soils from agrosystems, was tested [18–20]. The originality of the present study was in testing the impact of Cu on enzymatic equipment according to the fungal physiological state. Results of the present study showed that the response of extracellular enzymes to the metal was dose dependent in *T. versicolor*. The Cu addition at a geochemical level (20 ppm) slightly altered the secretion profiles of enzymes. At a higher contamination level (200 ppm), Cu modified the secretion profiles of hydrolases and oxidoreductases independently of physiological state of *T. versicolor*. Generally, the production of hydrolases was decreased by Cu, whereas that of oxidoreductases was highly increased. However, the sensitivity of enzymatic responses depended on the physiological state of the fungus and the type of secreted enzyme. A decrease in activities of cellulolytic hydrolases, including β-glucosidase, has also been observed in the presence of Cu in *Pleurotus ostreatus* [15]. Insofar as incubations of extracellular enzymes were performed in the presence of Cu, this decrease in hydrolase activities was not due to an interaction of Cu with enzymes at the extracellular level in the present study (data not shown). Among the tested hydrolases, only β-glucosidase gave a controversial response according to the exposure phase. Its activity was stimulated only when Cu was added during the stationary phase. This process may be due to cell lysis accentuated in the presence of the metal (Fig. 1), consequently releasing intracellular β-glucosidases. However, this was not observed for other hydrolases. An increase in ligninolytic oxidoreductases, such as Mn-peroxidase and laccase, has been reported in the presence of Cu in different filamentous fungi [13–16]. The present study showed that the exposure of *T. versicolor* results in a specific response of lignin peroxidase (Fig. 5d). This enzyme, not detected in control cultures, was specifically produced in the presence of Cu, which has not been reported in the literature. The stimulation of oxidase production can be explained by a metal action at the transcriptional level. Indeed, metal-responsive elements (MREs) have been characterized on the laccase gene in *P. ostreatus* [31] and on a gene cluster coding for Mn- and lignin peroxidases in *T. versicolor* [30]. Thus, it has been observed that RNA coding for a laccase is increased in the presence of Cu in *T. versicolor* [16]. With the presence of MRE on their genes, the oxidoreductases could be pertinent fungal biomarkers of metal exposure in more complex environments.

In summary, filamentous fungi represent one of the major biomasses in the terrestrial ecosystems and are essential for their functioning. Among these microorganisms, *T. versicolor* produces both hydrolases and oxidoreductases involved in the cycles of nutrient elements. The exposure of this fungus to Cu stress modulates the secretion of enzymes. Insofar as a metal stress can lead to functional modulations independently of the fungal physiological state, the response of these biochemical actors may be used as biomarkers of Cu contamination. The fact that oxidoreductases are preferentially secreted and highly stimulated during the metal stress makes these enzymes useful for this purpose. Finally, the present study constitutes a first step in understanding the regulation and secretion of functional systems of fungi and their response to a metal stress in order to improve enzymatic tools for ecotoxicity assessment. With inoculation of fungi specialized in oxidoreductase production in Cu-contaminated soils, further studies should allow assessment of the influence of complex properties of soil on these functional responses.

Acknowledgement—The present study was supported by the Institut National de la Recherche Agronomique and by the Conseil Régional de Haute Normandie.

REFERENCES

1. Nannipieri P, Kandeler E, Ruggiero P. 2002. Enzyme activities and microbiological and biochemical processes in soil. In Burns RG, Dick RP, eds, *Enzymes in the Environment*. Marcel Dekker, New York, NY, USA, pp 267–284.
2. Dick RP. 1997. Soil enzyme activities as integrative indicators of soil health. In Parkhurst CE, Doube BM, Gupta VVSR, eds, *Biological Indicators of Soil Health*. CAB International, Oxon, UK, pp 121–156.
3. Ascosta-Martínez V, Tabatabai MA. 2000. Enzymes activities in a limed agricultural soil. *Biol Fertil Soils* 31:85–91.
4. Kähkönen MA, Lankinen P, Hatakka A. 2008. Hydrolytic and ligninolytic enzyme activities in the Pb contaminated soil inoculated with litter-decomposing fungi. *Chemosphere* 72:708–714.
5. Kjøller AH, Struwe S. 2002. Fungal communities, succession, enzymes, and decomposition. In Burns RG, Dick RP, eds, *Enzymes in the Environment*. Marcel Dekker, New York, NY, USA, pp 267–284.
6. Bollag JM, Leonowicz A. 1984. Comparative studies of extracellular fungal laccases. *Appl Environ Microbiol* 48:849–854.
7. Orth AB, Denny M, Tien M. 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 5:2591–2596.
8. Bouws H, Wattenberg A, Zorn H. 2008. Fungal secretomes—Nature's toolbox for white biotechnology. *Appl Microbiol Biotechnol* 80:381–388.
9. Donnelly KC, Chen JC, Huebner HJ, Brown KW, Autenrieth RL, Bonner JS. 1997. Utility of four strains of white-rot fungi for the detoxification of 2,4,6-trinitrotoluene in liquid culture. *Environ Toxicol Chem* 16:1105–1110.
10. Mougin C, Jolivalt C, Briozzo P, Madzak C. 2003. Fungal laccases: from structure–activity studies to environmental applications. *Environ Chem Lett* 1:145–148.
11. Cajthaml T, Erbanová P, Kollmann A, Novotny C, Sasek V, Mougin C. 2008. Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*. *Fol Microbiol* 53:289–294.
12. Baldrian P. 2003. Interactions of heavy metals with white-rot fungi. *Enzyme Microb Technol* 32:78–91.

13. Crowe JD, Olsson S. 2001. Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Appl Environ Microbiol* 67:2088–2094.
14. Levin L, Forchiassin F, Ramos AM. 2002. Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii*. *Mycologia* 94:377–383.
15. Baldrian P, Valásková V, Merhautová V, Gabriel J. 2005. Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead, and zinc. *Res Microbiol* 156:670–676.
16. Collins PJ, Dobson ADW. 1997. Regulation of laccase gene transcription in *Trametes versicolor*. *Appl Environ Microbiol* 63:3444–3450.
17. Rama R, Sigoillot JC, Chaplain V, Asther M, Jolivalt C, Mougin C. 2001. Inoculation of filamentous fungi in manufactured gas plant site soils and PAH transformation. *Polycyclic Aromatic Compounds* 18:397–414.
18. Parat C, Chaussod R, Lévêque J, Dousset S, Andreux F. 2002. The relationship between copper accumulated in vineyard calcareous soils and soil organic matter and iron. *Eur J Soil Sci* 53:663–670.
19. Heijerick DG, Van Sprang PA, Van Hyfte AD. 2006. Ambient copper concentration in agricultural and natural european soils: an overview. *Environ Toxicol Chem* 25:858–864.
20. Laguerre G, Courde L, Nouaïm R, Lamy I, Revellin C, Breuil M, Chaussod R. 2006. Response of rhizobial populations to moderate copper stress applied to an agricultural soil. *Microb Ecol* 52:426–435.
21. Lesage-Meessen L, Delattre M, Haon M, Thibault JF, Ceccaldi BC, Brunerie P, Asther M. 1996. A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *J Biotechnol* 50:107–113.
22. Bradford MM. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254.
23. Nelson NJ. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* 153:375–380.
24. Seiboth B, Hartl L, Salovuori N, Lanthaler K, Robson GD, Vehmaanperä J, Penttilä ME, Kubicek CP. 2005. Role of the bga1-encoded extracellular β-galactosidase of *Hypocrea jecorina* in cellulase induction by lactose. *Appl Environ Microbiol* 71:851–857.
25. Keshri G, Magan N. 2000. Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusarium* spp. using volatile production profiles and hydrolytic enzymes. *J Appl Microbiol* 89:825–833.
26. Kim JH, Byun DS, Godber JS, Choi JS, Choi WC, Kim HR. 2004. Purification and characterization of arylsulfatase from *Sphingomonas* sp. AS6330. *Appl Microbiol Biotechnol* 63:553–559.
27. Wolfenden BS, Willson RL. 1982. Radical cations as reference chromogens in kinetic studies of one electron transfer reactions. *J Chem Soc Perkin Trans* 2:805–812.
28. Tien M, Kirk TK. 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proc Natl Acad Sci U S A* 81:2280–2284.
29. Paszczynski A, Huynh VB, Crawford R. 1986. Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. *Arch Biochem Biophys* 244:750–765.
30. Johansson T, Nyman PO. 1996. A cluster of genes encoding major isoenzymes of lignin peroxidase and manganese peroxidase from the white-rot fungus *Trametes versicolor*. *Gene* 170:31–38.
31. Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G, Sannia G. 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem J* 341:655–663.