Favouring the bioavailability of Zn and Cu to enhance the production of lignin-modifying enzymes in Trametes versicolor cultures

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Abstract

The metal effect on the enzyme secretion in fungi is usually related to total concentrations but not to bioavailable metal species. In this work, we aimed at enhancing the secretion of lignin-modifying oxidoreductases in *Trametes versicolor* by favouring the bioavailability of essential metals. For this purpose, the fungus was exposed to Cu or Zn in liquid culture media exhibiting different complexation levels. Metal speciation was determined experimentally or theoretically to quantify free metal species, supposed to be the most bioavailable, and species complexed to ligands. Although Zn^{2+} contents were high in media, Zn had no effect on the oxidoreductase production. Conversely, Cu highly induced the manganese peroxidase and laccase productions until 40 and 310 times when compared to unexposed controls. This inductive potential was highly correlated to Cu^{2+} contents in media. Furthermore, in lowly complexing media, the response threshold of oxidoreductases to Cu greatly decreased and an unexpected production of lignin peroxidase occurred.

Key words: metal bioavailability; free metal species; laccase; manganese peroxidase; lignin peroxidase.

1. Introduction

White-rot fungi are well known for their ability to degrade lignin, a recalcitrant

biopolymer widespread in the environment, and are thus fundamental for carbon flux in ecosystems. This unique ability of biodegradation results from the secretion of lignin-modifying enzymes by the fungi, namely manganese peroxidases (MnP), lignin peroxidases (LiP) or laccases (Hofrichter et al., 2010; Lundell et al., 2010). Because of the low specificity of their biocatalytic mechanisms, these extracellular enzymes mineralize a large range of organic contaminants (polycyclic aromatic hydrocarbons, pesticides, explosives, synthetic dyes…). Thus, it is assumed that these fungal oxidoreductases can be used as tools for waste treatment or bioremediation of contaminated soils or freshwaters (Mougin et al., 2003; Novotny et al., 2009). Furthermore, these fungal enzymes are used in various industrial bioprocesses such as biopulping or biobleaching (~~Lorenzo et al., 2002~~; Wang et al., 2006; Levin et al., 2010; Lundell et al., 2010). Considering these environmental and industrial applications of oxidoreductases, the enhancement of their production by fungi is of a great interest.

Although many organic compounds have been widely used to stimulate the production of fungal oxidoreductases (Mougin et al., 2002), the use of essential metals offers promising perspectives. Indeed, Cu increases the laccase production in different fungi (Crowe and Olsson, 2001; Baldrian, 2003; Lebrun et al., 2010). This response to metals has been explained at the transcriptional level. For example, Collins and Dobson (1997) showed that the stimulation of laccase activity by Cu corresponds to an increase in its mRNA in *Trametes versicolor*. Metal Responsive Elements (MRE) found in the promoters of genes enable the induction of their transcription in the presence of specific metals. The functionality of MRE has been demonstrated by the identification of transcription factors, proteins recognizing these elements, but only in some basidiomycetes like *Pleurotus ostreatus* or *Ceriporiopsis subvermispora* (Palanco et al., 2002; Faraco et al., 2003), Such MRE have been characterized on genes coding for a laccase in *Pleurotus ostreatus* (Giardina et al., 1999) and coding for MnP and LiP in *T. versicolor* (Johansson and Nyman, 1996). However, no assessment of LiP stimulation by metals has been reported whereas LiP is known to be produced by this fungus in the presence of veratryl alcohol (Collins and Dobson, 1995). This could be related to a response threshold not attained because of too small amounts of impacting and available metals for fungi. In the literature, the major metal species suggested as bioavailable and inductive of biological responses are free cationic species (Meylan et al., 2004;

~~Vigneault and Campbell, 2005 ;~ Giller et al., 2009; Thakali et al., 2010). It can be thus hypothesized that the intensity and sensitivity of the response of fungal oxidoreductases to some metals could be enhanced by favouring the metal bioavailability. However, in the studies reporting fungal exposures to metals in liquid culture media, the biological responses are related to total metal concentrations without taking into account to the metal speciation. As a consequence, specific and bioavailable metal species involved in the stimulation of oxidoreductases are not known in fungi.

A significant aspect concerning the secretion of lignin-modifying enzymes by fungi is the composition of the liquid culture media used. In the literature, culture media differ both in their C/N ratios and contents in nutriments which influence the behavior of fungi (Fomina et al., 2003; Dekker et al., 2007; Levin et al., 2010). The medium composition is also crucial for the metal bioavailability because of the presence of metal ligands. Tartaric acid and thiamine often used for fungal growth are known as ligands complexing metals (~~Jönsson et al., 1987~; Lesage et al., 1996; Stamatis et al., 2007). Yeast extracts commonly added in culture media are also suspected to complex metals (Gonzalez-Gil et al., 2003). These different media compositions lead to difficulties to compare the inductive potential of metals on the production of fungal oxidoreductases. Thus, the establishment of relationships between the production of lignin-modifying oxidoreductases and the presence of specific and inductive metal species is necessary to control the ability of fungi to secrete these enzymes.

In this study, we investigated the influence of metal speciation on both the sensitivity and selectivity of the oxidoreductase response to two essential metals, Cu and Zn, in an efficient lignin-degrading fungus. We tested the hypothesis that free metal species (i.e., Cu^{2+} and Zn^{2+}) are the most bioavailable species and main inducers of the production of laccases, MnP and LiP. For this purpose, *Trametes versicolor* was cultured in liquid media either rich or poor in metal ligands in order to vary their complexation level and thus, the metal bioavailability. Increasing metal concentrations were added in the culture media and the response of fungal enzymes was assessed. The metal speciation was determined experimentally when possible (for Cu) or computed with geochemical program to distinguish free metal species from metal species complexed to inorganic or organic ligands.

2. Materials and Methods

2.1. Medium compositions and culture conditions

Trametes versicolor ATCC 32745 was grown in four liquid culture media which differed in their content in organic ligands (Table 1). One medium called Lesa was made after Lesage et al. (1996), and was chosen for its richness in tartaric acid, known to complex metals. The other media contained no tartaric acid and were performed from Abadulla et al. (2000) who used it with success for cultures of *Trametes* strains. We modified the composition by decreasing the yeast extract contents from 5 to 2.5 and 0 g/L to give media called Aba5, Aba2.5 and Aba0 media respectively. Chemical components of media were purchased from Fischer and Sigma-Aldrich. According to the supplier, yeast extracts purchased from BD contained 54.1% amino acids, 10.9% total N, 3.3% phosphate, 3.2% potassium, 0.5% sodium, 0.4% chloride, 0.09% sulfate, 0.07% magnesium and 0.01% calcium. Total contents of Cu, Zn and Fe in yeast extracts were determined by atomic absorption spectrometry (Varian, SpectrAA 220). We found 0.01, 0.10 and 0.06 mg of Cu, Zn and Fe per g of yeast extracts respectively. Total organic carbon in these extracts (48.1%) was determined by TOC-analyzer (TOC-VCSN, Shimadzu).

A mycelium mat on agar plugs (10 mm diam.) was inoculated into 10 mL of liquid medium in 150 mL Erlenmeyer flasks. Cultures were incubated statically in the dark at 25 °C. After 3 days of incubation, 100 µL of $CuSO_4$ or $ZnSO_4$ solutions sterilized by filtration (0.2 µm pore size) were added into liquid cultures at final concentrations ranged from 10 nM to 1 mM. Controls were done without added metals. After 7 days of metal exposure, mycelia were harvested by a nylon screen (40 µm) and dried to determine the fungal biomass. Mycelium free cultures were filtered through a filter (0.2 µm pore size) and used for the measurements of extracellular activities. The experiments were carried out with three independent replicates per treatment.

2.2. Determination of Cu^{2+} contents in culture media

Contents in free copper species (i.e., Cu^{2+}) in culture media were determined using a copper-ion selective electrode (Ion Selective Electrode, ISE25Cu-9, Radiometer) coupled with a reference electrode (XR100, Radiometer) at 25 °C. A

calibration curve was established with 0.01, 0.1 and 1 mM $CuClO_4$ buffers in the presence of a constant ionic strength of 0.1 M. The background electrolyte of calibration buffers was $NaClO_4$ in the absence or presence of various contents in KCl used to adjust the Cl^- contents mimicking the molarities of each culture medium. Slopes of the electrode response obtained in calibrations were close to the theoretical Nernstian slope of 29.6 (29.3 +/- 0.8, n = 18, $R^2 = 0.99$). After an addition of 1 mM $CuSO_4$, the Cu^{2+} contents were determined in the various media in their initial states (i.e., in the absence of the fungus) or after 3 days of fungus incubation (i.e., the day of metal addition). In the last case, fungal mycelia were harvested from media before the metal addition.

2.3. Theoretical speciation of Zn and Cu using the program Soilchem

The geochemical program Soilchem (Sposito & Coves, 1991) was used to calculate the speciation of Zn or Cu, i.e. the percentages of metal species free or complexed to inorganic or organic ligands after the metal addition in the various culture media. The pH and total contents in organic and inorganic components of the culture media were input in program to compute speciation. The Soilchem program took into account the complexation constants of metals with the amino acids identified in the yeast extracts as given by the supplier.

2.4. Extracellular oxidoreductase activities

Laccase activity was measured by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) at 420 nm in a citrate/phosphate buffer (CPB; 0.1 M, pH 3.0) at 30 °C (Wolfenden and Willson, 1982). Manganese and lignin peroxidase activities were monitored respectively by the oxidation of 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one at 334 nm in CPB (pH 5.0) in the presence of $MnSO_4$ and H_2O_2 (Paszczynski et al., 1986) and the oxidation of veratryl alcohol at 310 nm in CPB (pH 3.0) in the presence of H_2O_2 (Tien and Kirk, 1984).

One unit of enzyme activity was defined as the amount of enzyme that catalyzed 1 µmol substrate in 1 min. The activities were measured in triplicate from extracellular filtrates and expressed in U per g dry weight of fungal biomass (U/g d.w.).

2.5. Statistical analysis

Means and standard errors (SE) were calculated from three independent sets for each variable measured: fungal biomass, pH of liquid medium, Cu^{2+} contents and extracellular oxidoreductase activities. Effects of C/N ratios, pH values and metal speciation on oxidoreductase activities were analyzed by Pearson's principal component analysis (PCA) involving 378 data per metal for six concentrations of metal added from 10 nM to 1 mM in each culture medium. Statistical analyses (t-test, regression, PCA) were performed by XLStat (Addinsoft).

3. Results and Discussion

3.1. Fungal growth in the different culture conditions

Trametes versicolor did not grow in the liquid media, Aba0. For the three other culture media, the biomass production and pH values of media during the fungus incubations are presented in Fig. 1. A decrease in pH about two units occurred until the 7^{th} day of incubation, where the fungus reached a stationary phase whatever the considered medium. The lowest pH was observed in Aba2.5 followed by Aba5 and Lesa. The biomass production of *T. versicolor* was greater in Lesa with the highest content in organic carbon (Fig. 1 and Table 1). After 10 days of incubation, the fungal biomass was 64 and 53% in Aba5 and Aba2.5 respectively of that measured in Lesa, despite a higher content in yeast extracts of these two Aba media compared to Lesa (Table 1).

The metal effect on the growth of *T. versicolor* was assessed by exposing the fungus to Cu or Zn from 10 nM to 1 mM for 7 days. The metal additions were performed at the 3^{rd} day of incubation when the fungal biomasses were similar between the media. Zn had no significant effect on the biomass production whatever the medium used. As for Zn, Cu had no effect on the biomass production, except for the highest concentration of 1 mM where the biomass was 10% less in low complexing media, Aba5 and Aba2.5 (data not shown). In our conditions, we concluded that *T. versicolor* is tolerant to essential metals whatever the composition of medium.

3.2. Metal speciation as affected by the medium composition

The experimental determinations of Cu^{2+} contents allowed us to assess the

complexation level of liquid culture media in the presence or absence of *T. versicolor*. In the absence of the fungus, Cu added at 1 mM was almost at 100% in the free form in Aba0. But as soon as yeast extracts or organic substrates were introduced in the culture media, the Cu complexation levels increased in this order Lesa > Aba5 > Aba2.5 (Table 2), which was consistent with the media composition. In the presence of the fungus, after 3 days of fungus incubation, Cu^{2+} contents were 1.5-fold higher in each media compared to that in media in their initial state before inoculating the fungus. That was probably due to the decrease in pH during the fungal growth. In the absence of the fungus, an artificial mimicked decrease in pH was performed to test the effect of pH on Cu^{2+} contents (Table 2). In each media, these contents were at least 2-fold higher compared to that in the presence of the fungus. Thus, not only pH but also fluxes of carbon and nutriments during the fungal growth may lead to the changes in metal speciation during the incubations. For example, white-rot fungi including *T. versicolor* are known to secrete organic acids and more particularly oxalic acids, leading to both the decrease in medium pH and immobilization of ionic metals (Makela et al., 2002; Fomina et al., 2005).

The effect of pH on metal speciation can be predicted through the use of a geochemical speciation program, but the impact of fungus growth on copper speciation is worse predicted. Experimental Cu^{2+} contents determined both in the presence or absence of *T. versicolor* were plotted against the theoretical Cu^{2+} contents calculated by the program. This plotting showed a difference between theoretical and measured free copper, with $[Cu^{2+}]_{theoretical} = 1.47\ [Cu^{2+}]_{experimental}$, probably due to the presence of components able to complex metals but not identified in yeast extracts and so, not input in the speciation program. However, the linear relationship was highly significant ($R^2 = 0.91$), which validated the theoretical speciation results for Cu. This speciation program allowed to predict the percentage of each metal species (contents in free metals and in metals complexed with inorganic or organic ligands) and to extrapolate our results to Zn, since no experimental Zn^{2+} determinations could be made as for Cu.

Theoretical Cu and Zn speciation were computed for all exposure conditions (from 10 µM to 1 mM) and for each used medium. Results showed that contents in free species were always lower for Cu than for Zn, and lower in the following order: Lesa < Aba5 < Aba2.5. Such results are related to the higher stability constants of

complexation with ligands for Cu than for Zn (Sposito and Coves, 1991). An example of theoretical Cu and Zn speciation is shown Fig. 2 for the case of the highest concentration of added metal. Free Cu fractions were about 1%, 5% and 13% of the total Cu content and free Zn fractions were about 13%, 28% and 74% of the total Zn content in Lesa, Aba5 and Aba2.5 respectively. The high complexing level of Lesa medium and its low contents in free metal species were due to the presence of tartaric acid. Indeed, metals were calculated as being mainly complexed with these organic ligands (> 95% for Cu and > 83% for Zn). In Aba5 and still further in Aba2.5, culture media without tartaric acid, the level of organic complexation attributed here to amino acids was much lower for Cu and negligible for Zn, then favouring inorganic and free species. Since Aba media differing only for their contents in yeast extracts, our results confirmed that yeast extracts have complexing properties as suggested by Gonzalez-Gil et al., 2003.

3.3. Metal effects on the production of oxidoreductases

The production of lignin-modifying oxidoreductases in *T. versicolor* unexposed or exposed to Zn or Cu at 1 mM is shown in Fig. 3. In the absence of metal, no basal production of LiP was detected. The medium composition slightly affected the basal productions of laccase and MnP. The main effect appears for the laccase production which was twice higher in Lesa than in Aba media (about 50 and 24 U/g d.w. respectively). This could be explained by differences in C/N ratios and N sources between Lesa and Aba media as suggested by several authors (Fomina et al., 2003; Dekker et al., 2007; Levin et al., 2010).

After 7 days of exposure, no significant effect of Zn on the oxidoreductase activities was observed whatever the medium used. By contrast, Cu increased the production of MnP and laccase where the intensity of response depended on the used medium (Fig. 3). When compared to the unexposed controls, the stimulation factors of Cu for laccase were 2, 140 and 310 in Lesa, Aba5 and Aba2.5 respectively. MnP was stimulated by Cu in the same way but with lower factors of 1.3, 16 and 41 in Lesa, Aba5 and Aba2.5 respectively.

In the literature, the average values of laccase production determined by oxidation of ABTS in classical culture media and in the presence of well-known organic

inducers are ranged between 1.5-15.8 U/mL in *Trametes* species (Mougin et al., 2002; Wang et al., 2006). In our conditions, the maximal value of laccase production was of 7650 U/g d.w. or 38.5 U/mL, confirming the high potential of Cu to stimulate the production of lignin-degrading enzymes in *T. versicolor*. It is worth noting that the ability of fungi to secrete oxidoreductases also depends on fungal species or strains. For example, in a classic medium amended both Cu and veratryl alcohol like organic inducer, the laccase activity reached 8.1 U/mL and 148.6 U/mL in 28 days-aged cultures of *T. versicolor* (var. *antarcticus*) and *T. trogii* respectively (Levin et al., 2010).

3.4. Sensitivity of the response of oxidoreductases

T. versicolor was exposed to total metal concentrations as low as 10 nM to assess the sensitivity of oxidoreductase response. In the lesa medium, no effect of Cu on the production of fungal oxidoreductases was observed for concentrations below 1 mM. LiP was specifically produced in the presence of Cu and only in the lowly complexing media, Aba5 and Aba2.5 (Fig. 4). In these media Aba, the thresholds of response to Cu for three oxidoreductases were lowered at 1 µM, except for LiP in Aba2.5 where the threshold was of 10 µM (Fig. 4). For each enzyme, the intensity of stimulation was at least 2-fold higher in Aba2.5 than in Aba5 for the same concentration of added Cu.

Although free species of Zn were always higher than that of Cu whatever the complexation level of medium, Zn had no effect on the production levels of lignin-modifying oxidoreductases unlike Cu. This result is consistent with the selectivity of oxidoreductase response to some metals due to the presence of MRE sequences on genes coding for these enzymes (Faraco et al., 2003). The stimulation of laccase production by Cu has been observed in other fungi (Crowe and Olsson, 2001; Baldrian, 2003). This may be explained by the fact that Cu is a constitutive element of the laccase catalytic site (Mougin et al., 2003). However, Jarosz-Wilkolazka et al. (2006) showed that the laccase activity was also increased by Cd in *Abortiporus biennis* and *Cerrana unicolor*. The stimulation of laccase by Cu has also been shown to correspond to a regulation at the transcriptional level in *T. versicolor* (Collins and Dobson, 1997). Furthermore, we showed that Cu also increased the production of MnP though this metal is not known as a constitutive element of this enzyme. LiP not detected in the control cultures was specifically produced in the presence of Cu when Cu availability

was increased. Although LiP has been characterized by molecular tools in *Trametes* strains (Johansson and Nyman, 1996; Miki et al., 2010), this enzyme has never been reported to be produced and stimulated by any metal to our knowledge.

3.5. Relationships between the metal species and the oxidoreductase production

Statistical analyses by PCA were made to assess the influence of various potential stresses on the production of oxidoreductases by *T. versicolor*, including for each used medium (i) the total metal content ii) the distribution of different metal species for each concentration of added metal iii) the C/N and iv) pH values (Fig. 5). No relationships between the C/N ratios and the production of oxidoreductases were established contrary to what is reported in the literature (Fomina et al., 2003; Dekker et al., 2006). However, in the case of laccase, the effect of the medium composition on its production varied of a factor 2, which is very low compared to production levels attained after exposures of *T. versicolor* to Cu in poorly complexing media (with of stimulation factors above 300). No significant influence of pH on the response of oxidoreductases was established, probably due to the fact that its decrease during incubations occurred the same way whatever the used medium (Fig.1). But it is clear that this decrease in pH implies the secretion of organic acids by fungi, affecting thus both the speciation and bioavailability of metals (Makela et al., 2002; Fomina et al., 2005).

Among the different fractions of Cu (i.e. total, free, inorganic and organic), the free fraction had the best inductive potential on oxidoreductase activities whatever the used medium. Indeed, the production levels of LiP, MnP and laccase were highly correlated with the Cu^{2+} contents: ($R > 0.98$, $R > 0.79$ and $R > 0.77$ respectively; $P < 0.0001$). The fraction of Cu complexed to inorganic ligands was, in a lower extent, correlated to the oxidoreductase production levels unlike the total and organic fractions. The following hierarchy could be established for the inductive potential of Cu species: free > inorganic complexed > organic complexed > total. If we took into account the fraction of Cu complexed to amino acids (Fig. 5); this specific organic complexed fraction was significantly correlated with the stimulation of LiP, MnP and laccase activities. Levin et al. (2010) showed that the presence of some amino acids in the

culture medium like glutamic acids can stimulate the laccase and MnP activities. However, the basal productions of these enzymes were not significantly different in Aba media differing only in their content in yeast extracts and so, in amino acids.

We clearly demonstrated that the effect of Cu on the production levels of lignin-modifying enzymes was not directly related to total metal contents. For this purpose, the determination of free species contents is more relevant to predict the induction of biological responses and metal toxicity as suggested by other authors (Meylan et al., 2004; ~~Vigneault and Campbell, 2005;~~ Giller et al., 2009; Thakali et al., 2010). However, our results did not exclude other metal species than free species, such as Cu complexed to inorganic ligands or amino acids, participate to the response of oxidoreductases. Although the metal speciation and bioavailability are not taken in account during the exposures of fungi to metals, this point is consistent with assumptions of Degryse et al. (2006) made in plants. Indeed, the authors reported that metals, such as Zn or Cu, complexed to organic ligands can be available to plants because of their lability and their possible dissociation in contact to biological membranes.

4. Conclusions

Our results illustrate the relevance of taking account the metal speciation rather total contents to understand the fungal responses during metal exposures in liquid culture media. The feasibility of enhancing greatly the ability of fungi to secrete the lignin-modifying enzymes thanks to essential metals is demonstrated. Decreasing the metal complexation level of culture media and thus, favouring the bioavailability of metal allow to increase the sensitivity and intensity of oxidoreductase responses which are selective to some metals, such as Cu. It is thus possible to overproduce fungal enzymes without losing biomass for industrial and environmental applications by employing an essential element in low amounts.

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Table 1. Composition of liquid media used for the cultures of *T. versicolor*.

	Medium			
Nutrient (g/L)	**Lesa**	**Aba5**	**Aba2.5**	**Aba0**
maltose	20	-	-	-
glucose	-	10	10	10
tartarate di-NH_4	2.3	-	-	-
NH_4Cl	-	2.5	2.5	2.5
yeast extracts	1.0	5.0	2.5	-
tartarate di-Na	1.8	-	-	-
KCl	-	0.5	0.5	0.5
KH_2PO_4	2.0	2.0	2.0	2.0
$CaCl_2 \cdot 2H_2O$	0.1	0.1	0.1	0.1
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5	0.5
$FeSO_4 \cdot 7H_2O$	0.07	-	-	-
total C	9.4	4.4	3.2	2.0
total N	0.2	1.2	0.9	0.7
C/N	37.9	3.7	3.5	3.1

Table 2. Influence of the *T. versicolor* growth and pH on Cu^{2+} contents determined by Cu-ISE in culture media after adding of Cu at 1 mM.

	Cu^{2+} contents (µM)		
Medium	**Lesa**	**Aba5**	**Aba2.5**
In the absence of *T. versicolor*	3.1	4.2	32.3
	± 0.1	± 0.2	± 1.1
(*constant initial pH*)	(*5.5*)	(*5.4*)	(*5.5*)
In the presence of *T. versicolor*	4.2	6.6	51.3
	± 0.3	± 0.1	± 7.9
(*pH after 3 days incubation*)	(*4.8*)	(*4.8*)	(*4.4*)
In the absence of *T. versicolor*	9.3	17.5	87.9
	± 0.4	± 0.8	± 4.0
(*pH adjusted to that of the 3rd day of incubation)*	(*4.8*)	(*4.8*)	(*4.4*)

Fig. 1. Biomass production (closed symbols) and pH values (open symbols) during the incubation of *T. versicolor* in various liquid culture media: Lesa (diamonds), Aba5 (squares) and Aba2.5 (triangles). The values are means ± SE for triplicate cultures.

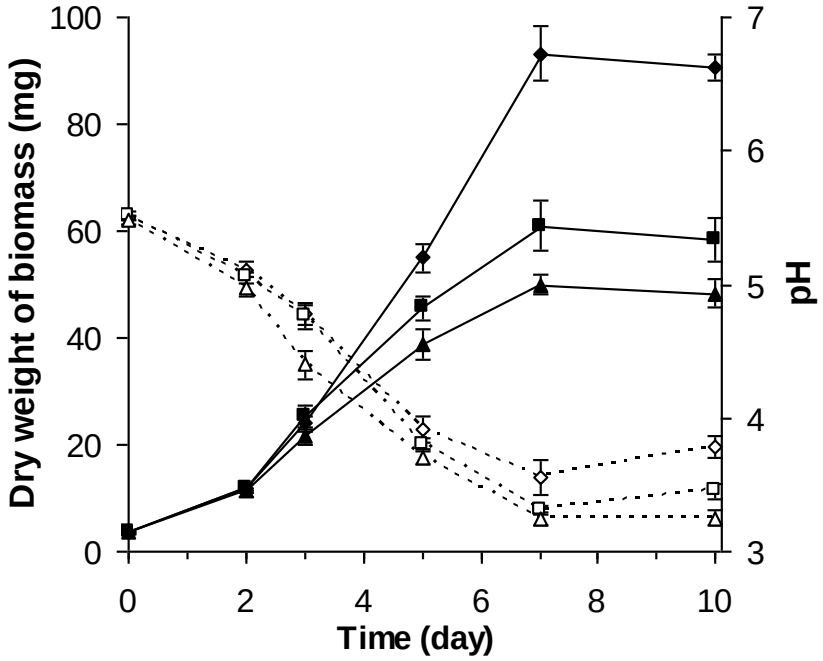

Fig. 2. Distribution of organic-complexed (■), inorganic-complexed (■) and free (□) species of Cu (A) or Zn (B) added at 1 mM in the different culture media. The percentages were calculated using the Soilchem program.

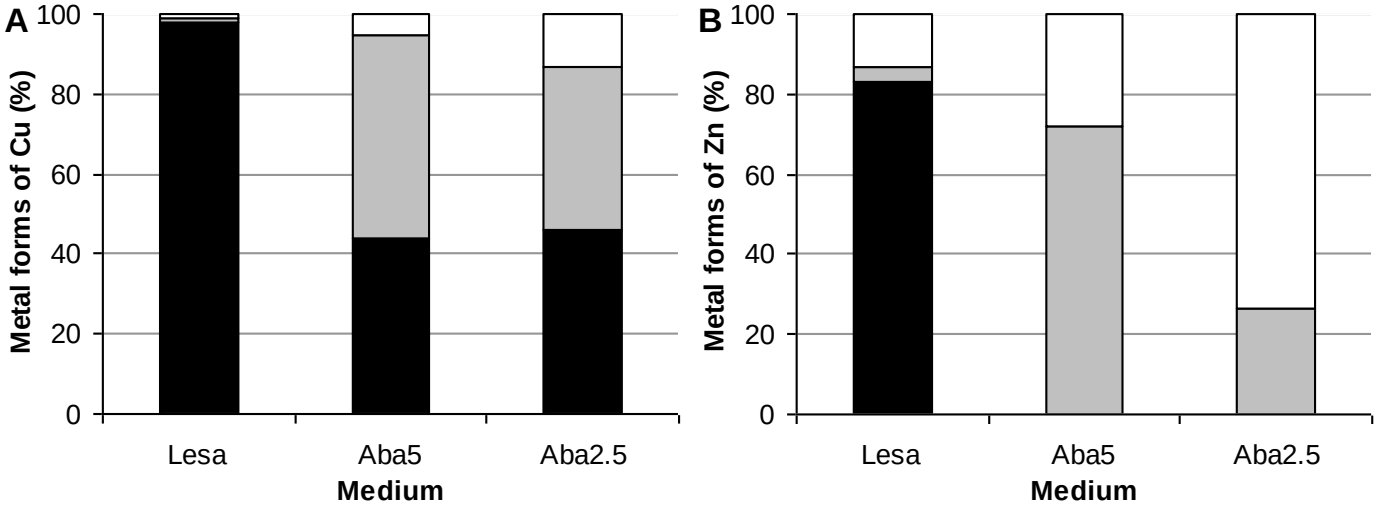

Fig. 3. Laccase and manganese peroxidase activities in liquid cultures of *T. versicolor* unexposed (□) or exposed for 7 days to Zn (■) or Cu (■) at 1mM in different culture media. The values are means ± SE for triplicate cultures. * Significant differences compared to the respective unexposed controls for each medium ($P < 0.05$).

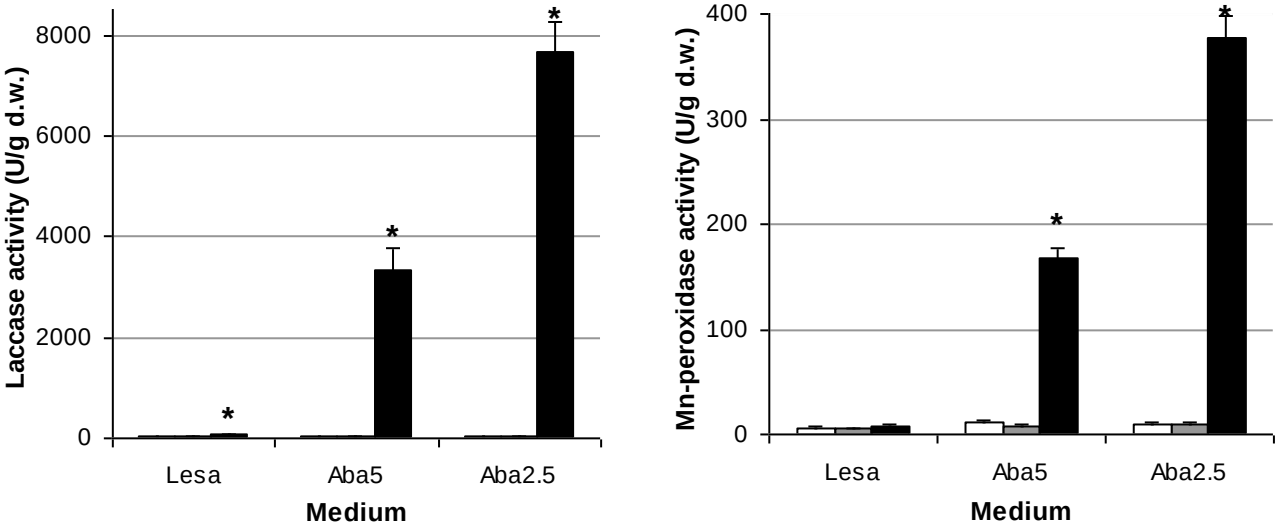

Fig. 4. Responses of extracellular oxidoreductases by *T. versicolor* exposed for 7 days to Cu in Aba5 (□) or Aba2.5 (■). The values are means ± SE for triplicate cultures. Significant differences: * and **, compared to the respective unexposed controls for each medium ($P < 0.05$).

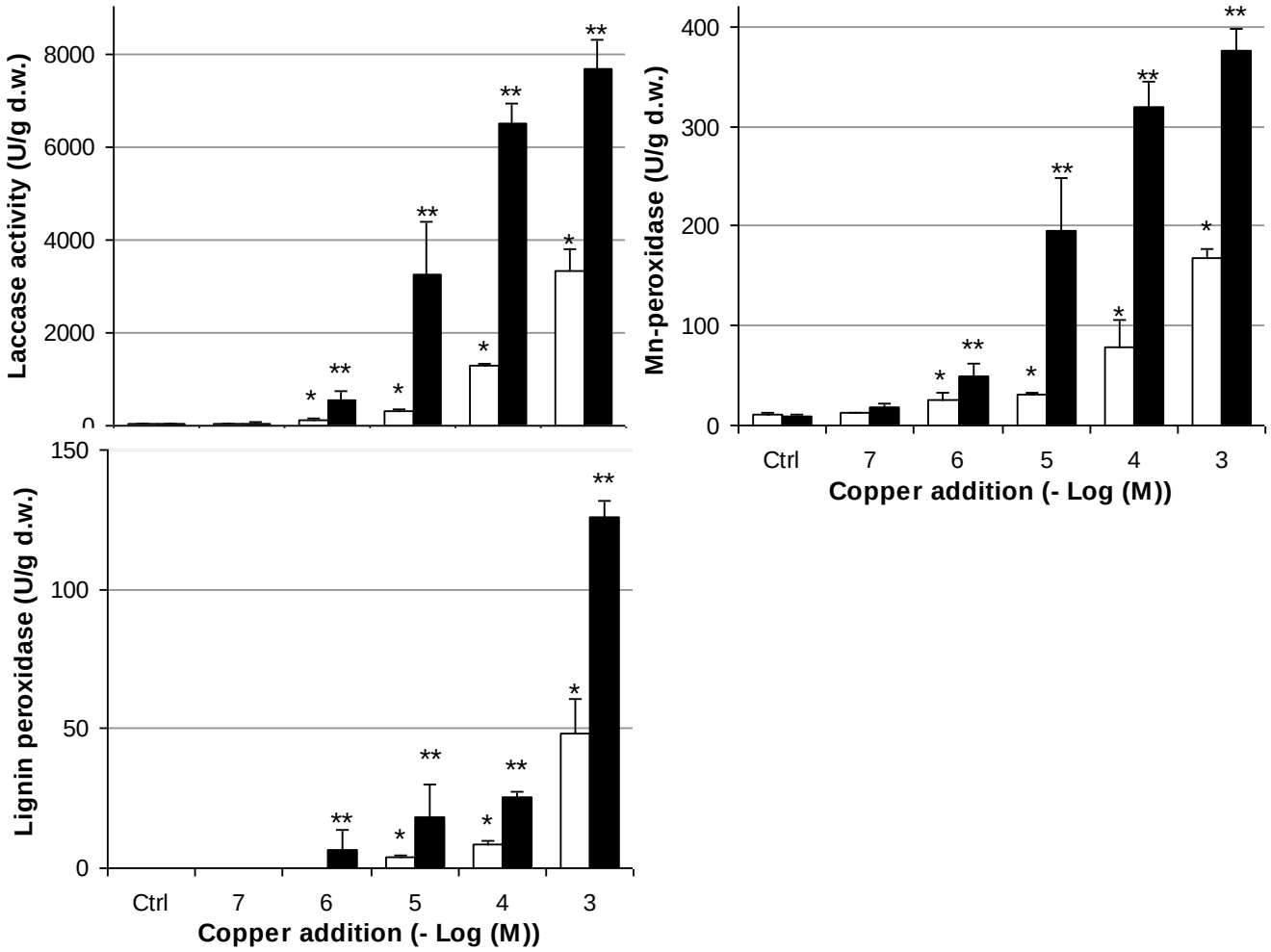

Fig. 5. Correlation circles of Pearson's principal component analysis between the different fractions of Cu (total, organic, inorganic and free), the C/N ratio, pH and stimulation of oxidoreductase activities by Cu. This analysis concerns six concentrations of Cu (10 nM to 1 mM) added in Lesa, Aba5 and Aba2.5 media.