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ORIGINAL PAPER

Effect of chemical and metallic compounds on biomass, mRNA levels and laccase activity of *Phlebia brevispora* BAFC 633

María Isabel Fonseca · Ana Belén Ramos-Hryb · Julia Inés Fariña · Silvana Soledad Sawostjanik Afanasiuk · Laura Lidia Villalba · Pedro Darío Zapata

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Abstract Nine aromatic compounds (caffeic acid, syringaldehyde, vanillic acid, guaiacol, vanillin, sinapic acid, syringol, syringic acid and ferulic acid) and four metallic compounds ($CuSO_4$, $AgNO_3$, $MnSO_4$, and $CaCl_2$) were tested for their ability to increase laccase (Lac) activity in the ligninolytic basidiomycete *Phlebia brevispora* BAFC 633. The addition of syringaldehyde, syringol, guaiacol, sinapic acid, vanillin, ferulic acid and $CuSO_4$ showed a positive effect on fungal growth; however, it decreased dramatically with the addition of $AgNO_3$ and did not undergo changes in the presence of $CaCl_2$ or $MnSO_4$. Lac activity increased with the addition of all the compounds tested, depending on the concentration and the day of culture. *P. brevispora* BAFC 633 produced two isoenzymes, a constitutively expressed of 60 kDa and another of 75 kDa expressed upon induction by sinapic acid, $MnSO_4$ or $CuSO_4$. Lac secretion capacity of *P. brevispora* BAFC 633 can be increased 27 times higher than the control with the highest levels detected in the presence of 0.3 mM $CuSO_4$ at day 14. The action is affected at pre-transcriptional level regulating at the onset of the process, however it does not rule out the effect at the post-transcriptional and post-translational levels, for which is necessary to deepen in the knowledge of all possible regulation points of gene expression.

Keywords Laccase · White rot fungi · *Phlebia brevispora* BAFC 633 · Isoenzymes · Induction

M. I. Fonseca (✉) · A. B. Ramos-Hryb · S. S. Afanasiuk · L. L. Villalba · P. D. Zapata
Laboratorio de Biotecnología Molecular, Módulo de Bioquímica y Farmacia, Facultad de Ciencias Exactas Químicas y Naturales, UNaM, Av. Mariano Moreno 1375 (3300), Posadas, Misiones, Argentina
e-mail: biotecmol2010@gmail.com

J. I. Fariña
Departamento de Biotecnología Fúngica, PROIMI-CONICET, T4001MVB San Miguel de Tucumán, Argentina

Introduction

White-rot-fungi basidiomycetes possess the ability to completely mineralize lignin, a complex biopolymer which is very recalcitrant to degradation (Buswell and Odier 1987; Kirk and Farrell 1987). They are the most effective lignin-degrading microbes in nature through the action of three major classes of enzymes designated lignin peroxidases (LIPs), manganese dependent peroxidases (MNPs), and laccases (Lac), which play an important role in the fungal degradation of lignin (Kirk and Farrell 1987).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates (Thurston 1994). This enzyme is generally extracellular and catalyzes the oxidation of several phenolic compounds, aromatic amines, thiols and some inorganic compounds using molecular oxygen as electron acceptor (Pezzella et al. 2009). The low substrate specificity makes this enzyme interesting for biotechnology purposes in various industries such as decolorization and detoxification of textile dyes and effluents, pulp delignification, removal of phenolics from wines and other beverages, transformation of antibiotics, steroids and many aromatic compounds (Mayer and Stapler 2002).

The regulation of the expression of the Lac gene differs from one organism to another, probably reflecting the

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different physiological roles that these enzymes play in the fungi (Collins and Dobson 2002; Mansur et al. 1998).

Extracellular Lac from Basidiomycetes are produced constitutively in small amounts (Bollag and Leonowicz 1984) although their production can be stimulated by the presence of inducers (mainly phenolic compounds or aromatic related to lignin or lignin derivatives).

Many studies are focused on the optimization of Lac activity by searching for the type, concentration and the suitable addition time of the low molecular weight compounds that could act as inducers as well as the culture medium supplements to stimulate Lac activity (Galhaup et al. 2003). Some studies have shown that Lac activity is induced when aromatic compounds (veratryl alcohol, vanillic acid, 2,5-xylidine, ferulic acid, syringaldazine and guaiacol) or metal ions (Cu^{2+}) are added to the culture medium (Ikehata et al. 2004), although this effect should be checked for each particular strain.

Some of the Lac-encoding genes have also been cloned and genetically characterized (Saparrat et al. 2010; Mäkelä et al. 2006; Xiao et al. 2006). Promoter regions of Lac genes usually contain typical promoters such as xenobiotic and metallic compounds (Piscitelli et al. 2011; Xiao et al. 2006; Faraco et al. 2003; Soden and Dobson 2001), but there are differences among promoter regions of the genes of relevant Lac isoforms in the same fungal strain that justify studies on potential inducers.

Fonseca et al. (2010, 2013) and Fonseca (2012), reported high levels of Lac of *Phlebia brevispora* BAFC 633. In the present work we evaluate the effect of aromatic compounds and metallic ions on Lac activity, isoenzymes composition and biomass growth on culture of *P. brevispora* BAFC 633.

Materials and methods

Organism and culture conditions

Phlebia brevispora BAFC 633 was provided by the Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. Stock cultures were maintained on malt agar at 4 °C.

Growth conditions and induction of laccase

One agar plug (36 mm^2) of *P. brevispora* BAFC 633 grown on MEA plates was inoculated into 50 mL liquid medium in a 250 mL Erlenmeyer flasks. Liquid medium contained 12.7 g/L malt extract and 5 g/L corn step liquor 5.0. The initial pH was adjusted to 4.5 with HCl 0.1 N before sterilization. The culture was incubated at 29 °C under static condition. The metallic compounds; $CuSO_4$, $AgNO_3$, $MnSO_4$, $CaCl_2$ were added individually under aseptic conditions.

The 3-day-old cultures of *P. brevispora* BAFC 633 were treated with aromatic compounds; sinapic acid, syringic acid, syringol, syringaldehyde, vanillic acid, vanillin, guaiacol, caffeic acid, ferulic acid and mixed with the growing medium. The effect dose/response was evaluated at three different concentrations; 0.02, 0.1 and 0.3. All the samples were incubated at 29 °C under static condition and collected at 7, 10 and 14 days of cultivation.

Biomass and protein determination

Biomass growth was determined by measuring the mycelium dry weight, while proteins were measured by the Bradford method on the conditioned medium.

Liquid media was separated from the supernatant mycelia by filtering in a Büchner funnel using fiberglass filters (GF/C) and frozen at −20 °C until use. Biomass dry weight was determined by the difference between the fiberglass filters (GF/C) weight before and after filtration through a Büchner funnel and subsequent drying at 80 °C till constant weight.

Protein was determined by micro-test using the Bradford technique (BioRad) following manufacturer's instructions with bovine serum albumin as the standard (Bollag and Leonowicz 1984).

Enzyme assays

Lac (EC 1.10.3.2) activity was determined by changes in the absorbance at 469 nm ($E_{469} = 27.5\ mM^{-1}\ cm^{-1}$) in a spectrophotometer Shimadzu UV-3600, using 2,6-dimethoxyphenol (DMP) as substrate. The assay mixture contained 5 mM of DMP in 0.1 M sodium acetate buffer (pH 3.6) and a suitable amount of enzyme, which was incubated at 30 °C (Field et al. 1993).

The enzyme activity was expressed as international units (U), defined as the amount of Lac which gives an increase of 1 μmol product/min at 30 °C. The activity was reported in U/L.

Statistics analysis

Two-way ANOVA with Bonferroni post test was performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Polyacrylamide gel electrophoresis

Cell-free filtrates were subjected to native polyacrylamide gel electrophoresis (ND-PAGE, 7.5 % w/v). After proteins

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separation, the gel was incubated in 0.1 M sodium acetate buffer containing 5 mM DMP for Lac activity detection (Fonseca et al. 2010). After 5-min incubation, the dye solution was discarded and the gel was immediately scanned with a Scanner HP Deskjet F300 All-in-One series. In order to determine Lac isoenzymes molecular weight, an electrophoretic separation by SDS-PAGE (7.5 % w/v), followed by a subsequent renaturation and detection technique was performed as previously described in the literature (Murugesan et al. 2007; Fonseca et al. 2010, 2013) and compared to a molecular weight marker (Kaleidoscope, BioRad).

The secreted protein profile was observed using SDS-PAGE analysis performed on 12 % (w/v) polyacrylamide gel (Laemmli 1970). Proteins were stained with 0.2 g/L silver (Eggert et al. 1996) using a Biorad mini VE vertical electrophoresis system (Bio-Rad, CA, USA). The electrophoretic run was performed at 100 V for 120 min in 1.5 M Tris–Glycine buffer (pH 8.3).

Isolation of total RNA

Mycelium from a liquid grown culture was harvested, washed twice with cold 0.1 M Tris, 0.02 M EDTA, subsequently digested with a mixture of lysis buffer (GuSNC 4 M, Tris 0.1 M, EDTA 0.02 M Triton X 100 1 %) for 20 min. A sterile dipstick was then used to break down the tissue.

The purification was performed using phenol saturated in phenol and chloroform. The proteins were precipitated with potassium acetate 3 M and the RNA was precipitated with isopropyl alcohol, washed with ethanol, dried and resuspended in RNases-free sterile water (Chomczynski and Sacchi 1987). To confirm the quality of the isolated RNA, 0.8 % agarose gels test was performed. RNA was quantified by spectrophotometer technique at 260 nm.

Amplification of Lac fragment by PCR

Samples were treated with DNases previous to retrotranscription step. Double-stranded cDNA syntheses were carried out at 42 °C for 60 min in 13 µL containing Tris–HCl pH 8.3 250 mM, KCl 250 mM, $MgCl_2$ 20 mM, DTT 50 mM, dNTPs 2.8 mM of each one, ARNsin 20 U, 10 pmol of oligo-dT and MMuLV-Transcriptase Invers (Fermentas) 1 U.

The primers used for PCR technique were previously described by D'Souza et al. (1996) for Lac from other basidiomycetes (Luis et al. 2004).

The PCR technique was carried out with 20 µL containing KCl buffer 1×, $MgCl_2$ 2.5 mM, dNTPs 200 µM, 10 pmol of each primer, 0.5 U Taq polymerase and 25 ng of DNA. PCR cycling was 4 min 94 °C, 35 cycles of 40 s 94 °C, 40 s 50 °C, 40 s 72 °C and final extension of 10 min at 72 °C.

The results of the amplification were verified by electrophoresis in gels of agarose at a concentration of 2 %, stained with ethidium bromide and photographed with ACER digital camera. To confirm the PCR specificity, the amplicons were sequenced.

Results

Effect of the addition of aromatic compounds on biomass production and protein secretion

Phlebia brevispora BAFC 633 is characterized by a homogeneous growth in liquid culture with slightly whitish translucent coloration. Typical growth of *P. brevispora* BAFC 633 was observed in most of the treatments, however with the addition of guaiacol, vanillin, $AgNO_3$ and syringol a change of mycelium color from white to pink was detected (the intensity of the color produced is proportional to the inducer concentration).

Ferulic acid treatment induced higher fungus growth, reaching a maximum at day 14 with 0.02 and 0.1 mM ($p < 0.001$). However, there were no significant differences in the levels of secreted proteins in any of the treatments ($p > 0.05$) (Fig. 1, ferulic acid).

The guaiacol tests showed an increase in biomass with 0.3 mM from day 10 ($p < 0.05$). However, there were no significant differences in protein secretion ($p > 0.05$) (Fig. 1, guaiacol).

In trials with syringic acid there were no significant differences in fungal growth ($p > 0.05$), but the quantity of secreted proteins, reaching a maximum at day 14 with 0.3 mM ($p < 0.05$) (Fig. 1, syringic acid).

The trials with vanillic acid showed significant differences in biomass production at day 14 for the three concentrations, 0.02 ($p < 0.01$) and 0.3 and 0.1 mM ($p < 0.001$). Furthermore, secreted proteins increased with the addition of 0.3 mM of vanillic acid at 14th culture day ($p < 0.05$) compared to the control (Fig. 1, vanillic acid).

In trials with vanillin maximum biomass production and protein secretion were observed at day 14 of treatment with 0.3 mM ($p < 0.05$) (Fig. 1, vanillin).

Caffeic acid produced significant differences on biomass production at 14 days of cultivation at the three concentrations tested ($p > 0.05$), whereas protein secretion showed no changes ($p > 0.05$) (Fig. 1, caffeic acid).

With sinapic acid there was a significant increase only in biomass at day 14 with 0.3 mM ($p < 0.01$), whilst no significant differences in the levels of secreted proteins ($p > 0.05$) were detected (Fig. 1, acid sinapic).

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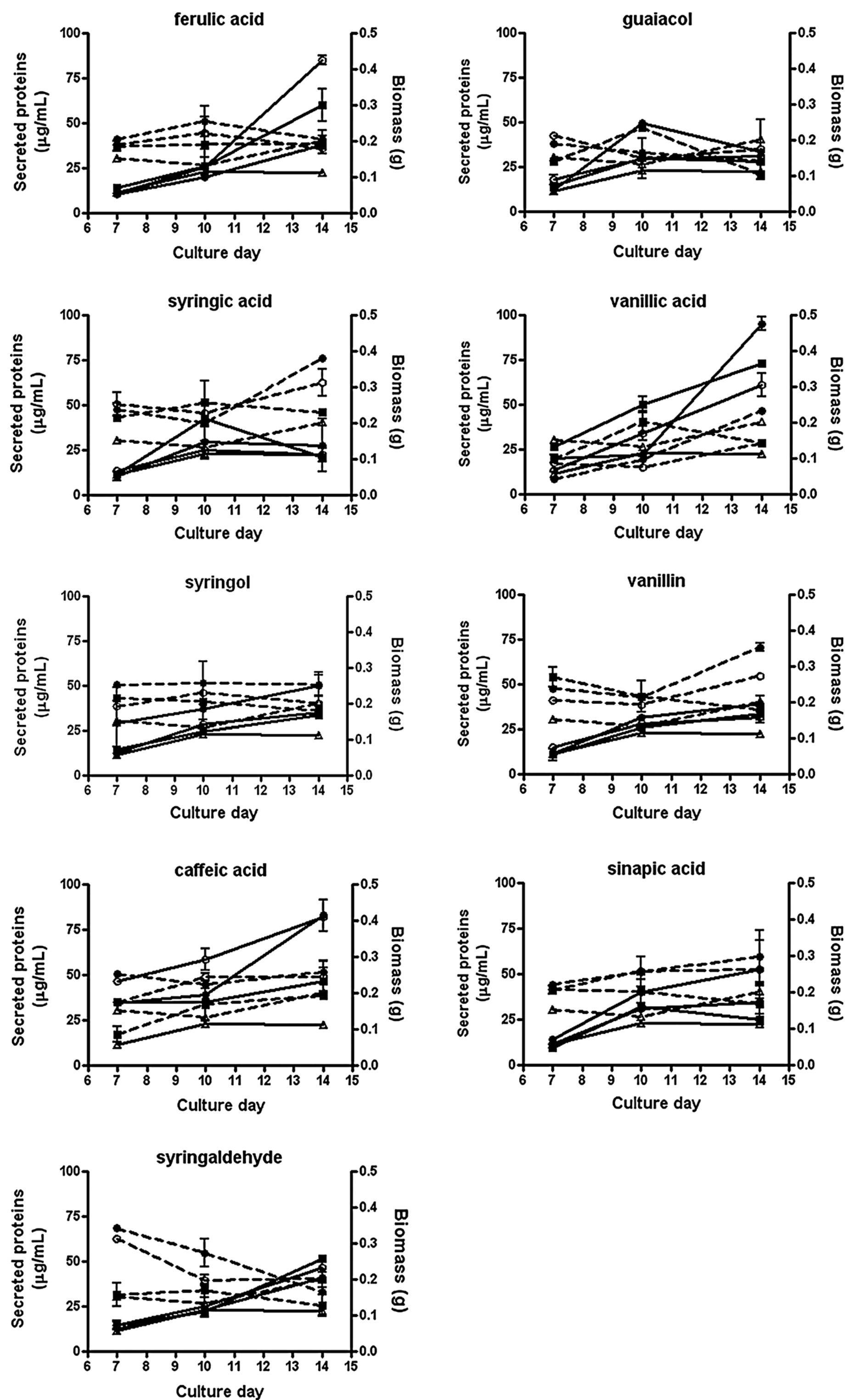

Fig. 1 Effect of aromatics compounds on biomass production (*solid line*) and secreted proteins (*dashed line*) in *P. brevispora* BAFC 633 at days 7, 10 and 14 of culture with 0.02 mM (*square*), 0.1 mM (*open circle*), 0.3 mM (*filled circle*) and without the compound (*triangle*). The biomass corresponded to 50 mL of culture

In trials with syringaldehyde there were significant differences in the values of biomass production at day 14 of culture with 0.02 mM ($p < 0.001$), 0.1 mM ($p < 0.01$) and 0.3 mM ($p < 0.05$). Regarding protein secretion, there was a marked increase at day 7 with 0.1 and 0.3 mM concentration ($p < 0.05$) (Fig. 1, syringaldehyde).

Syringol treatment showed a maximum mycelial growth at day 14 with 0.3 mM ($p > 0.05$), however, there were

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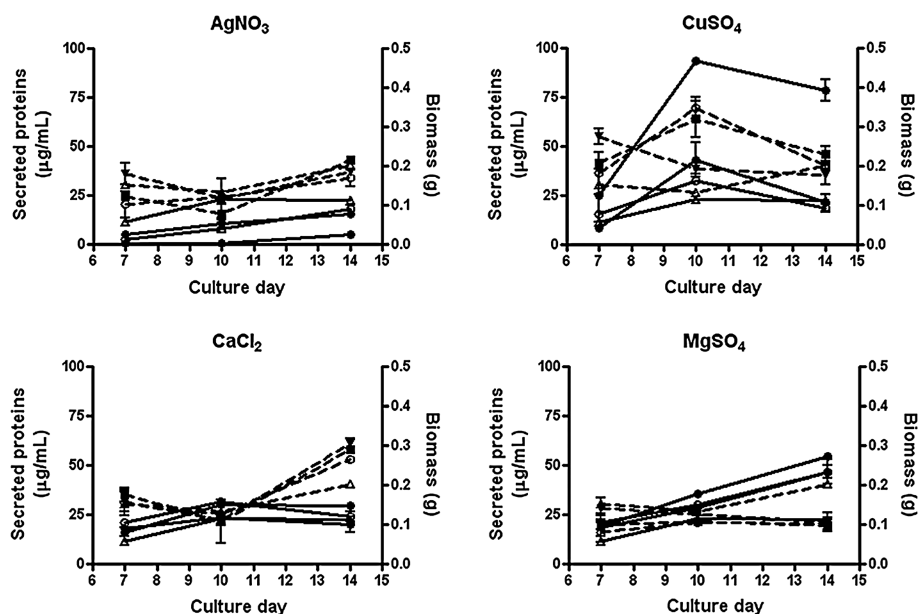

Fig. 2 Effect of metal ions on biomass production (*solid line*) and secreted proteins (*dashed line*) on *P. brevispora* BAFC 633 on 7, 10 and 14 of culture days with 0.02 mM (*square*), 0.1 mM (*open circle*) and 0.3 mM (*filled circle*) and within metal (*triangle*). The biomass processing corresponded to 50 mL of culture

no significant differences regarding protein secretion ($p > 0.05$) (Fig. 1, syringol).

Effect of the addition of metallic ions on biomass production and protein secretion

The $AgNO_3$ produced a dramatic decrease in growth starting day 7 of culture with 0.1 mM ($p < 0.05$) and 0.3 mM ($p < 0.01$), and starting at day 10 with 0.02 mM ($p < 0.01$). However, there were no significant differences in terms of protein secretion in any of the treatments tested ($p > 0.05$) (Fig. 2, $AgNO_3$).

$CuSO_4$ treatment produced maximum mycelial growth at day 10 of culture with 0.3 mM ($p < 0.05$). Secreted protein levels showed a significant increase at day 10 of culture in both trials with 0.02 mM and 0.3 mM ($p < 0.05$) (Fig. 2, $CuSO_4$).

Moreover, trials with $MnSO_4$ and $CaCl_2$ showed no statistically significant differences in the levels of biomass or in secreted protein levels (Fig. 2, $MnSO_4$ and $CaCl_2$).

Effect of the addition of aromatic compounds on Lac activity and isoenzyme profile

The syringic acid treatments resulted in an increased Lac activity, which was detectable from day 7 at the highest concentration tested (0.3 mM, $p < 0.001$) and more evident at day 14 at all the concentrations ($p < 0.001$). Furthermore, the electrophoretic profile of the zymograms revealed the presence of a unique enzyme fraction with all the treatments and culture times (Fig. 3, syringic acid).

Vanillic acid also induced an increase in enzyme activity for all treatments ($p < 0.01$). The zymogram showed one type of Lac at all the concentrations and different culture times (Fig. 3, vanillic acid).

Sinapic acid addition caused an increase in the Lac activity, which was particularly evident at day 14 with a maximum at a concentration of 0.3 mM ($p < 0.01$). The zymogram revealed a major band of Lac, where the presence of two isoenzymes was evidenced with 0.02 mM at day 14 of culture (Fig. 3, sinapic acid).

Vanillin treatments increased Lac activity in all concentrations, with the highest stimulation at day 14 ($p < 0.01$). The zymograms showed a single type of Lac in all assays (Fig. 3, vanillin).

Regarding treatment with caffeic acid, there was an increase of enzyme activity from day 7 at all the concentrations ($p < 0.05$). The zymograms evidenced only one type of Lac with these culture conditions (Fig. 3, caffeic acid).

Regarding the treatment with syringol, the increase in Lac activity was noted in most of the concentrations tested, becoming more significant at day 14 of culture, especially with 0.02 and 0.1 mM ($p < 0.01$), and also 0.3 mM ($p < 0.05$). The zymogram revealed a single type of Lac for all treatments and culture times tested (Fig. 3, syringol).

The addition of 0.3 mM ferulic acid or guaiacol to the cultures showed the maximum increase in Lac activity at all days evaluated ($p > 0.05$), whereas syringaldehyde showed the maximum effect at day 14 at the lowest concentration tested (0.02 mM, $p < 0.05$). In all these cases the zymogram revealed a single type of Lac (Fig. 3).

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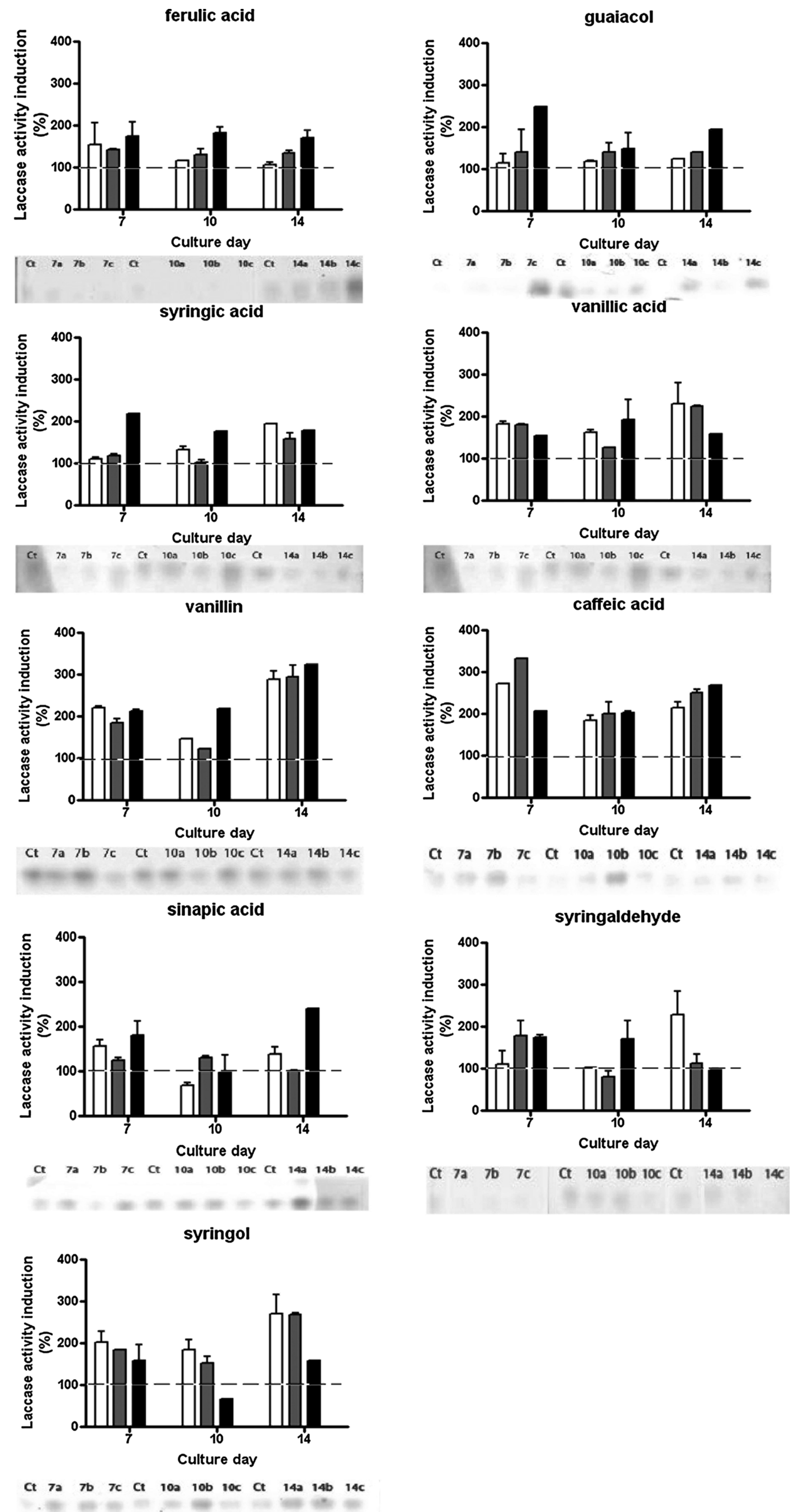

Fig. 3 Effect of different concentrations of aromatics: 0.02 mM (*white bar*), 0.1 mM (*grey bar*) 0.3 mM (*black bar*) and without the compound (*dashed line*), on Lac activity of *P. brevispora* BAFC 633. Below each graph the zymogram of Lac activity from supernatants is shown. 20 µg protein/well were seeded for each condition tested, using gels at 7.5 % w/v ND-PAGE revealed with DMP. Control (Ct) samples without the compound. The *numbers* indicate the days of cultivation and the *letters* the compounds concentration (*a*) 0.02, (*b*) 0.1 y (*c*) 0.3 mM. Data are representative of three independent experiments

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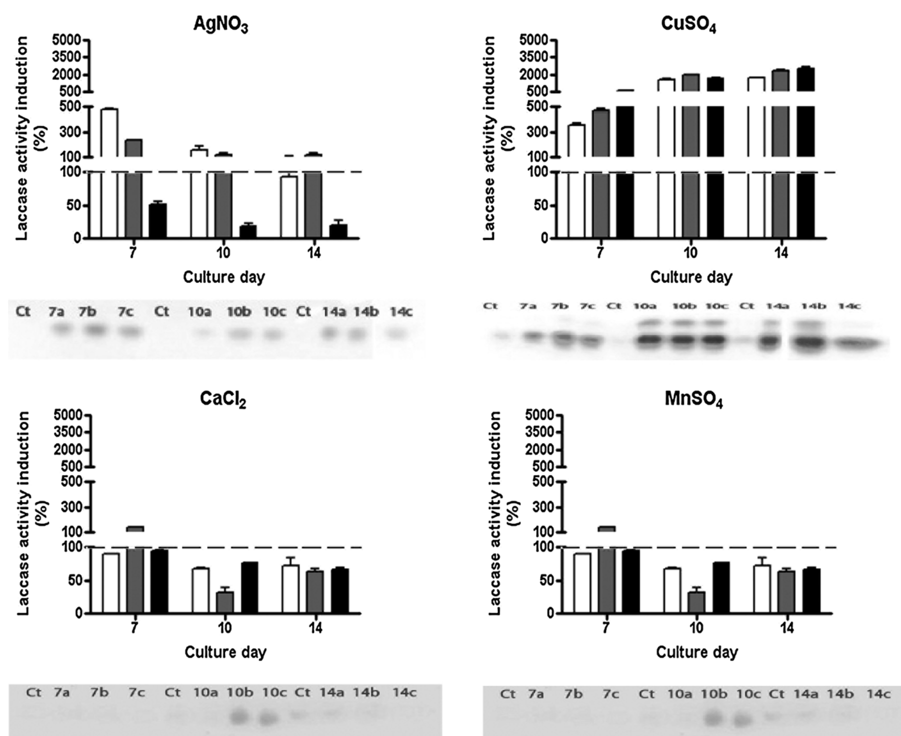

Fig. 4 Effect of different concentrations of metal compounds with 0.02 mM (*white bar*), 0.1 mM (*grey bar*) 0.3 mM (*black bar*) and without the compound (*dashed line*), on Lac activity of *P. brevispora* BAFC 633. Below *each graph* the zymogram from Lac supernatants is shown. 20 µg protein/well were seeded for each condition tested, using gels at 7.5 % w/v ND-PAGE revealed with DMP. Control (Ct) samples without the compound. The *numbers* indicate the day of cultivation and the *letters*, the compounds concentration (*a*) 0.02, (*b*) 0.1 y (*c*) 0.3 mM. Data are representative of three independent experiments

Effect of the addition of metal ions on Lac activity and isoenzyme profile

The enzyme activity values showed a significant increase with 0.02 and 0.1 mM $AgNO_3$, at day 7 of culture ($p < 0.001$ and $p < 0.01$, respectively). However, enzyme production was significantly decreased in the assays with 0.3 mM ($p < 0.05$). In the zymogram results, the presence of a single enzyme in all the treatments was evidenced (Fig. 4, $AgNO_3$).

In the test with the addition of $CuSO_4$, the Lac activity revealed a progressive increase with the concentration of the metallic compound and incubation time with the maximum at day 14 with 0.3 mM, which represents a value around 27 times higher than the control ($p < 0.001$). The zymogram revealed the presence of two Lac isoenzymes (Fig. 4, $CuSO_4$). As presented by Fonseca et al. (2010), the addition of 0.5 mM $CuSO_4$ resulted in the stimulation of Lac activity of 49.2 times, which demonstrate that the effect is dependent on the concentration of Cu^{2+} and a value of 0.5 of $CuSO_4$ mM would be more appropriate to obtain greater stimulation of its production.

Regarding treatment with $MnSO_4$, there was an overall increase in Lac activity at various concentrations tested, maximum with 0.3 mM at 14 days of cultivation ($p < 0.001$). The zymogram showed two Lac types, one of which was observed in the test with 0.01 mM at day 14 of culture only (Fig. 4, $MnSO_4$).

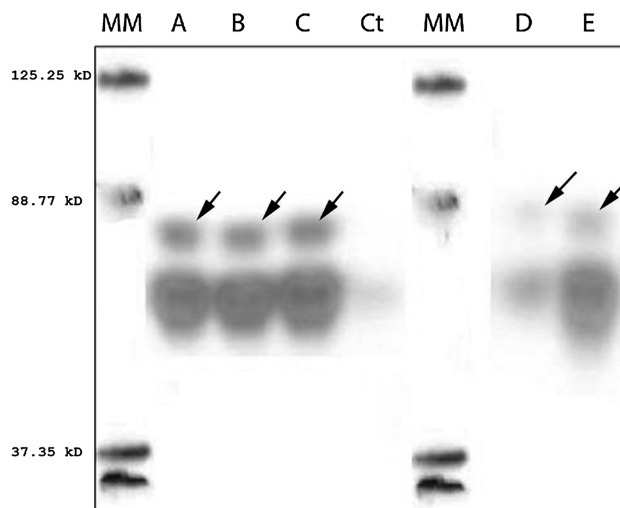

Fig. 5 Estimation of molecular weight (SDS-PAGE) of Lac isoenzymes of *P. brevispora* BAFC 633, produced on ME supplemented with various compounds. 20 µg secreted protein/well at day 10 with 0.02, 0.1 and 0.3 $CuSO_4$ (*A*, *B*, *C*) without copper (Ct) at day 14 with 0.02 mM sinapic acid (*D*) at day 14 with 0.1 mM $CuSO_4$ (*E*), were analyzed by SDS-PAGE gels revealed with 7.5 % DMP. Inducible Lac isoenzymes are indicated with arrows. Data are representative of two independent experiments

Regarding the addition of $CaCl_2$, it was detected a decrease of Lac activity for the various concentrations tested, the effect becoming more pronounced with culture time. The zymogram profile showed a single Lac type at all conditions (Fig. 4, $CaCl_2$).

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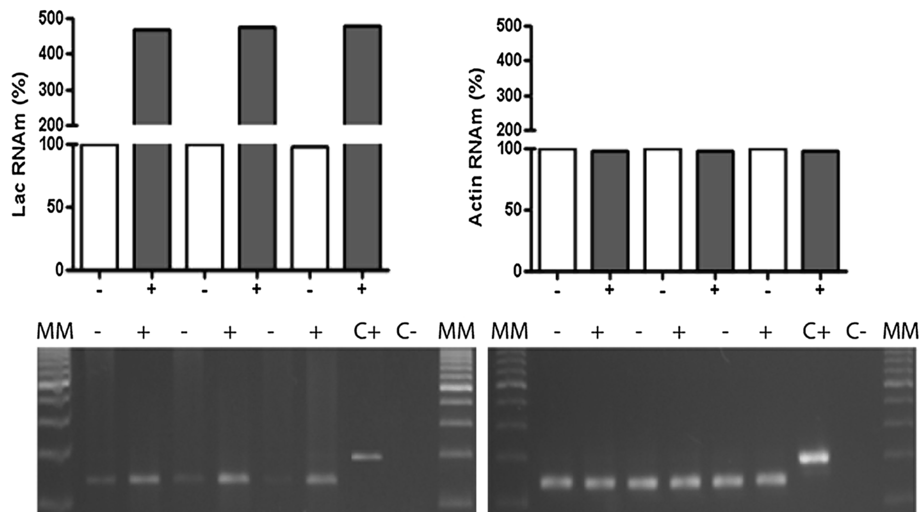

Fig. 6 Semiquantitative RT-PCR for mRNA of Lac (*left*) and actin mRNA (*right*). Three independent experiments were performed with RNA isolated from mycelium grown in ME in absence (−) or presence of 0.5 mM $CuSO_4$ (+). The densitometry graph is shown above and the matched products of RT-PCR separated using electrophoresis in agarose 2 % (w/v) are shown below). The positive control (C+ corresponds to the 200 bp fragment obtained from genomic DNA (amplification control) and negative control (C−) corresponds to the amplification without the presence of cDNA or genomic DNA respectively

To confirm the molecular weight of the different isoenzymes observed in previous experiments, SDS-PAGE technique was applied to obtain the electrophoretic separation (Fig. 5).

The presence of a constitutive isoenzyme of 60 kDa, in control and in all treated samples (Figs. 3, 4 and 5) was clearly detected. The 75 kDa isoenzyme, was missing in the control samples, but was detected in assays with $CuSO_4$ at day 10 for the 3 concentrations tested, and on day 14 with 0.02 to 0.1 mM. This second isoenzyme was also observed in the test with 0.02 mM sinapic acid and 0.1 mM $MnSO_4$ at day 14. Both isoenzymes showed variations at different concentrations of inductors and analyzed on the day of cultivation.

Laccase gene expression of *P. brevispora* BAFC 633 on the presence of copper

Biochemical studies hitherto showed that there are many factors that determine the levels of Lac activity in *P. brevispora* BAFC 633 at different culture conditions, but the most marked increase was registered with the addition of copper. For this reason there was an interest to study the possible effect of this metal at Lac gene level.

As shown in Fig. 6, Lac mRNA increased significantly (five times) when copper was added to the culture medium, which is due to increased levels of Lac gene transcription. An actin mRNA fragment was used as a control reaction, whose gene transcription levels remained unchanged in all treatments.

The secreted total protein levels were increased significantly in the presence of $CuSO_4$. The SDS-PAGE gels showed an increase of all protein fractions especially between 35 kDa and 80 kDa, in the range which is the Lac *P. brevispora* BAFC 633 described in this paper (Fig. 7).

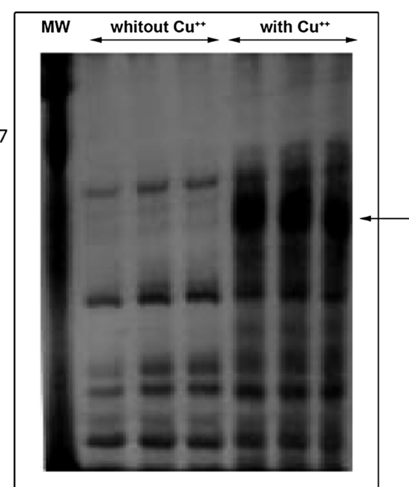

Fig. 7 Protein profile obtained from culture supernatants of *P. brevispora* BAFC 633 without $CuSO_4$ and with 0.5 mM $CuSO_4$ using SDS-PAGE 12 % w/v. *All lanes* were seeded with 20 µg of total protein. Lac is indicated with *arrow*

Discussion

A change in mycelial color and morphology in the cultures supplemented with guaiacol, vanillin, syringol and $AgNO_3$ was clearly observed.

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Since metals can be attached to the cell walls (Horikoshi et al. 1981), this effect can be useful as an efficient and economical way to remove toxic metals from dilute aqueous solutions by biosorption.

Phlebia brevispora BAFC 633 supplemented with vanillic acid, ferulic acid, sinapic acid, syringol, syringaldehyde, guaiacol and vanillin promoted mycelia growth. The increases in biomass were observed in *Phanerochaete chrysosporium* with vanillin, vanillic acid and caffeic acid (De la Rubia et al. 2002), in *Trametes pubescens* and *Cyathus bulleri* with guaiacol (Galhaup and Haltrich 2001; Salony and Bisaria 2006). The $CuSO_4$ was found to be a strong stimulator of mycelial growth in *P. brevispora* BAFC 633. Copper is an essential heavy metal for fungal growth, a micronutrient and also an activator of several enzymes in fungal and pigment synthesis. On the other hand, high concentrations of copper can strongly inhibit fungal growth (Levin et al. 2002; Baldrian 2003; Patel et al. 2009). Intrinsic properties that determine the survival in the presence of metals include pigmentation in cellular walls, extracellular polysaccharides and excretion of metabolites. Some fungi with the ability to reduce the toxicity of Cu^{2+} or other metals, immobilize ions through the intracellular production and extracellular of chelating compounds such as organic acids and siderophores (Guillén and Machuca 2008), but this tolerance to copper decreased when the concentration is above 30 mM.

$AgNO_3$ reduces growth in *P. brevispora* BAFC 633, which could be because this metal can be toxic to the strain (Baldrian 2003), and is not one of the essential elements for fungal growing (Gadd 1993). The inhibitory effects of the metal were also observed in *T. pubescens* (Galhaup and Haltrich 2001).

Regarding the values of secreted proteins, only treatments with syringic acid, vanillin and syringaldehyde showed significant differences on protein production in *P. brevispora* BAFC 633. These results were observed with vanillin in *Lentinula edodes* (Cavallazzi et al. 2005), however, vanillic acid caused a decrease in protein secretion. In *Aspergillus niger* a deficiency of manganese caused a reduced level of lipids, proteins and nucleic acids (Kubicek et al. 1979; Orthofer et al. 1979). The literature also reported decreases caused by copper in *P. ostreatus* (Palmieri et al. 2000), probably due to the action of certain proteases secreted in the medium containing copper. Copper and cadmium, and aromatic amino acid residues may also caused oxidative damage to proteins by inducing oxidative stress associated with the production of reactive complexes (Baldrian 2003).

Syringic acid is a metabolite from the fungi ligninolytic degradation (Chen et al. 1982) that acted as an inducer of Lac activity in the case of *P. brevispora* BAFC 633 and in *Ganoderma lucidum*, Lac secretion was increased three times (D'Souza et al. 1999).

Previous investigations have shown that aromatics acids of low molecular weight are structurally related to the phenolic fractions of lignin and serve as good inducers of ligninolytic enzyme (Eggert et al. 1996; Yaver and Golightly 1996).

Low concentrations of vanillic acid acted as inductor in *P. brevispora* BAFC 633. In *Cerrena unicolor* the best inducers were 2,4,6-trinitrotoluene > vanillic acid > pyrogallol (Elisashvili et al. 2009) and also vanillic acid in a medium containing ethanol, but the addition of mannitol suppressed Lac synthesis. These data lead to the assumption that the inducing effect depends of other aromatic compounds present in the culture medium. In *Pleurotus eryngii* (Muñoz et al. 1997) and *C. unicolor* MTCC 5159 (D́Souza-Ticlo 2008) the vanillic acid stimulated Lac activity. Furthermore, in *Abortiporus biennis*, *C. unicolor* and *Trametes versicolor*, the vanillic acid induced Lac activity in lower degree compared to other inducers (Cho et al. 2006). It has been found that the addition of the compound has no effect on enzyme activity in *Pleurotus ostreatus* (Palmieri et al. 2000). In other cases, such as in *T. pubescens* MB89, the addition reduced the Lac activity (Galhaup et al. 2003).

Our results showed that ferulic acid is not a good inducer of enzyme activity in *P. brevispora* BAFC 633. In other fungus, such as *T. versicolor*, however, proved to be a strong inducer (Cho et al. 2006; Minussi et al. 2002), *Trametes villosa* (Minussi et al. 2002), *T. pubescens* (Galhaup et al. 2003), *Pycnoporus cinnabarinus* SS3 (KoroIjova-Skorobogat'ko et al. 1998), *Trametes* sp. I-62 (Terron et al. 2004), *C. unicolor* MTCC 5159 (D́Souza-Ticlo 2008).

In *A. biennis*, *C. unicolor* and *T. versicolor* the guaiacol had no significant increase in Lac enzymatic activity (Cho et al. 2006). However, several authors have reported the positive effect of guaiacol addition as in *P. ostreatus* (Patel et al. 2009), *C. bulleri* (Salony and Bisaria 2006), *T. versicolor* (Lee et al. 1999), *Trametes* sp. I-62 (Terron et al. 2004), *Coriolopsis fulvocinerea* (Gorbatova et al. 2006), *C. unicolor* MTCC 5159 (D́Souza-Ticlo 2008) and *Coriolus hirsutus* (KoroIjova-Skorobogat'ko et al. 1998). These findings demonstrate the existence of differences in the response to various inducers on each of the treated fungal species and that every new isolate need to be explored.

The induction with sinapic acid in *P. brevispora* BAFC 633 was also observed in *T. versicolor* (Cho et al. 2006).

Syringaldehyde was not a good inducer of enzyme activity in strains of *T. versicolor*, *A. biennis* and *C. unicolor* (Cho et al. 2006).

Several studies (Thurston 1994, Youn et al. 1995) suggested that one of the most important roles of Lac is to

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protect against phenolic compounds produced as a result of the degradation of lignin, which may be toxic to the fungal body. However, the contradictions between the results showed that the protection role of Lac still remains unclear.

Caffeic acid increased Lac activity in *P. brevispora* BAFC 633 as in *P. cinnabarinus* SS3 (KoroIjova-Skorobogat'ko et al. 1998) but did not modify the levels in *Sclerotium rolfsii* (Ryan et al. 2003). The syringol increased the enzymatic activity in *P. brevispora* BAFC 633 as in *Lentinus strigosus* (Myasoedova et al. 2008).

The negative effect of $AgNO_3$ on *P. brevispora* BAFC 633 may be due to a gradual accumulation of the compound until reaching toxic concentrations for the cells. Baldrian (2003) indicated that the heavy metals generally are strong inhibitors of enzymatic reactions. Heavy metals may also affect other chemical reactions in the cell, regulating by means of other path the biodegradation process of fungus. Furthermore, the activation or inhibition of proteolytic enzymes by metals can also change the balance of extracellular enzymes (Palmieri et al., 2000).

Another metal that has been reported as a potential inductor of Lac activity is Ca^{2+} (Murugesan et al. 2010). In this study we observed that the addition of $CaCl_2$ to the culture media produced a slight increase in Lac activity of *P. brevispora* BAFC 633. It has been proposed that Lac induction responds to oxidative stress (Fernández-Larrea and Stahl 1996; Rollins and Dickman 1998). This could be detected directly by means of a transcriptional activation or indirect activation caused by pathways of calcium and thermal shock due to disruption of membrane protein and disarming.

Just as in *P. brevispora* BAFC 633, in *T. pubescens* both copper and manganese (1 mM) increased Lac activity (Galhaup and Haltrich 2001).

$CuSO_4$ is an excellent inducer of Lac activity in *P. brevispora* BAFC 633. Several authors have investigated the effect of the addition of this metal at various concentrations (Galhaup et al. 2003; Stajic et al. 2006; Patel et al. 2009), and their results are also consistent with the present work. Copper is an essential nutrient for fungal growth and functions as an activator of several fungal metal enzymes (Jellison et al. 1997).

The inducible isoenzyme was observed with the addition of $MnSO_4$, $CuSO_4$ and sinapic acid to *P. brevispora* BAFC 633, and is both dose and time dependent. This suggests the possibility of differential post-translational regulation for each chemical agent and in the presence of several genes coding for each Lac isoenzyme (Xiao et al. 2003). *P. ostreatus* in the presence of copper showed three Lac isoenzymes (POXA1b, and POXC POXA2) and *T. versicolor* two isoenzymes (Lac I and Lac II) (Palmieri et al. 2000). *P. ostreatus* revealed different isoenzyme patterns (Palmieri et al. 2000) with $MnSO_4$, in *Coprinus comatus* an also reported the induction of different Lac isoenzymes (Lu and Ding 2010).

With respect to the molecular masses, the Lac molecule typically ranges between 60 and 80 kDa (Thurston, 1994) and may appear different due to variations in glycosylation of the enzyme (Xiao et al. 2003, 2004). In the case of *P. brevispora* BAFC 633 strain, molecular weights of the isoforms (60 and 75 kDa) were consistent for the three inductors.

Many Lac genes respond to metals promoters (Yaver and Golightly 1996; Faraco et al. 2002). In *P. brevispora* BAFC 633, the Cu^{2+} is a regulator of the expression of Lac at transcriptional level evidenced by increases in the mRNA levels. Similar work conducted with *T. villosa* showed increases in mRNA levels too, which denoted the inducting effect of copper at the transcriptional level (Yaver and Golightly 1996; Palmieri et al. 2000; Preussler et al. 2009). Here it was demonstrated that there is a direct correlation between the increase of mRNA levels and the increase of enzyme activity in cultures added with copper, in agreement to previous work (Soden and Dobson 2001). These findings suggest that the Cu^{2+} regulates the Lac expression at the transcriptional level possibly by binding to MRE (metal response element). However, metals may also act stabilizing mRNA or activating the processing, trafficking and protein folding. In favor of this, the induction with non-metallic compounds such as ferulic acid in strains of *P. ostreatus* (Palmieri et al. 2000), showed an increase in the level of Lac mRNA transcript, without expressing significant enzyme activity.

For these reasons and based on the evidence provided by the experimental work conducted, we can conclude that the Cu^{2+} acted at pre-transcriptional level regulating the beginning of the process, even though we cannot exclude the role at the post-transcriptional and post-translational levels. It is certainly necessary to deepen on the knowledge of all possible points of regulation of gene expression.

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Conflict of interest All authors declare no conflicts of interest.

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