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Full Length Research Paper

Molecular genetic identification and extracellular laccase activity of two wild fungal isolates from Mexico

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Fungal strains BM-1 and BG-2 were isolated from rotting stemwood of the *Ipomoea murucoides* tree. Both strains were assigned to *Botryosphaeria rhodina* (teleomorph)/*Lasiodiplodia theobromae* (anamorph) complex by the internal transcribed spacer (ITS) sequence of Nuclear Ribosomal Unit. BM-1 produced 4.39 ± 0.35 µg/mL, and BG-2 3.61 ± 0.28 µg/mL of extracellular protein in non-induced culture. BM-1 increases this amount by 93% when $CuSO_4$ (150 µM) is added to medium, and by 73% after addition of ethanol (10% v/v). Corresponding increases for BG-2 were of less than 40%. Basal activity of extracellular laccase had a maximum value of 0.379 ± 0.042 U/mL for BM-1 and of 0.312 ± 0.130 U/mL for BG-2. $CuSO_4$ (150 µM) addition to the culture increased the maximum activity of laccase up to 3.964 ± 0.385 U/mL in BM-1 and up to 5.270 ± 0.0.793U/mL in BG-2. Ethanol addition (10% v/v) to the culture increased the maximum activity of laccase to 3.639 ± 0.506 U/mL in BM-1 and to 11.397 ± 0.440 U/mL in BG-2. The physiological differences observed between strains BM-1 and BG-2, stresses the importance of characterizing wild isolates from new substrates with the objective of finding strains of biotechnological potential.

Key words: *Botryosphaeria* wild strains, ITS DNA sequence, extracellular laccase.

INTRODUCTION

Extracellular laccases produced by filamentous fungi involved in white rot are part of the multienzymatic complex in charge of the degradation of lignocellulosic residues in nature (Hatakka, 2001; Leonowicz et al., 2001; Martínez et al., 2005). That enzymatic activity has been widely studied in basidiomycete fungi (Baldrian, 2006), but has also been reported in ascomycetes and their anamorphs (Iyer and Chattoo, 2003; Liers et al., 2006; Kellner et al., 2007; Forootanfar et al., 2011).

Extracellular laccases can catalyze the oxidation of a variety of phenolic and non-phenolic substrates (Wong, 2009), which makes possible to use the enzyme in different biotechnological processes, including some applications in the food (Minussi et al., 2002) and textile (Riva, 2006; Arora and Sharma, 2010) industries, in bioremediation processes (Mayer and Staples, 2002; Mougin et al., 2003) and in the synthesis of organic molecules and biomaterials (Mikolasch and Schauer, 2009). That´s why interest exists in the isolation and characterization of wild fungi strains having extracellular laccase activity (Levin et al., 2004; Dritsa et al., 2007; Barrasa et al., 2009). Additionally, the isolation and characterization of new strains of filamentous fungi that produce extracellular laccases is important given the potential of these strains to provide enzymes having different substrate affinities or optimal activities over a wide range of pH and temperature, which would allow for optimization of industrial applications of the enzyme or for generating new biotechnological processes.

Identification of fungal wild isolates is important in order to fully exploit all its biotechnological potential and to contribute to diversity description and conservation (Chiu

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et al., 2000; Lee et al., 2006; Brock et al., 2009). The objectives of the present work are to make a molecular genetics characterization of two strains of wild fungi isolated from rotting stem wood of the tropical tree *Ipomoea murucoides* Roem. et Schult. (Convolvulaceae) and to evaluate the production of extracellular laccase of these isolates.

MATERIALS AND METHODS

Culture media

The potato dextrose agar (PDA) medium (Difco, USA) was used for the isolation and maintenance of vegetative mycelium and for the generation of inocula for culture in liquid medium. The medium was sterilized at 121°C (15 lb/in^2). The potato dextrose broth (PDB) medium (Difco, USA) supplemented with 5 g/L of yeast extract was used for the study of basal and induced extracellular enzyme activity. Enzyme activity was induced by addition to the culture medium of either $CuSO_4$ (150 µM) or ethanol (10% v/v).

Isolation of studied strains

Samples of rotting stemwood of *Ipomoea murucoides* Roem. et Schult. (Convolvulaceae) were collected in the states of Guanajuato and Michoacán (in Central West Mexico). Stemwood samples were placed in sterile plastic bags and processed for strain isolation on the same day of collection. Fragments of approximately 1.0 cm in length were placed in Petri dishes with PDA medium supplemented with ampicillin and streptomycin at a concentration of 50 mg/L and tannic acid at a concentration of 0.5 g/L. Inoculated Petri dishes were incubated at 28°C until vegetative mycelium growth was observed, whereupon, colonies were reseeded in the same medium using a 5 mm diameter plug taken from the peripheral initial colonies. Three consecutive reseedings were made until verification of the establishment of axenic cultures.

DNA extraction, PCR assay, and sequencing

High molecular weight DNA was extracted from vegetative mycelium from strains inoculated in PDA medium following the method described by Liu et al. (2000). The ITS region of the Nuclear Ribosomal Unit was amplified using the oligonucleotide pair ITS1/ITS4 and the protocol described by White et al. (1990). Amplification products were analyzed in 2.0% agarose gels stained with ethidium bromide (Sambrook and Russell, 2001). The BigDye Terminator v3.1 kit (Applied Biosystems Inc., USA) was used for the sequencing PCR following the provider instructions. Sequencing reactions were performed in ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

Analysis of obtained sequences

The obtained sequences were compared with Genbank sequences by means of the algorithm Blastn. Sequences in the Genbank showing higher similarity with the sequences obtained in the present work were selected to make a multiple alignment using the algorithm Clustal W in the software package MEGA 4 (Tamura et al., 2007), editing the sequences to optimize the alignment by means of the program BioEdit (Hall, 1999).

In order to select the algorithm for the calculation of genetic distances between the obtained sequences, a new alignment was generated from the edited sequences after which an analysis of nucleotide substitution pattern was made using the software FindModel in the page http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html. The Kimura 2-parameter algorithm was among the best models found for describing the nucleotide substitution patterns, because of which it was used for computing the genetic distances using MEGA 4. Finally, a clustering pattern was obtained from the calculated genetic distances by means of the Neighbor-Joining algorithm using MEGA 4.

Growth kinetics

Growth kinetics in liquid medium and assays of enzymatic activity, were made with three 5 mm diameter plugs from the periphery of cultures grown in PDA medium at 28°C in logarithmic growth phase that were used for inoculation of 50 mL of PDB in 250 mL flasks, which were incubated at 28°C and agitation at 120 rpm. Growth was determined by measurement every 24 h of dry weight of mycelium recovered by filtration. All kinetic determinations were made by triplicate and the results of measurements were averaged, calculating the standard deviation for each point in the growth curve.

Assessment of extracellular laccase activity

Determination of extracellular laccase activity was made following the procedures of Nagai et al. (2002). Briefly, the reaction mix was composed of 500 µL McIlvaine buffer at pH 4.0 (0.1 M citric acid, 0.2 M sodium phosphate dibasic), 300 µL sterile distilled H_2O, 100 µL ABTS (2, 2´- azino-bis (3- ethylbenzothiazoline- 6- sulphonic acid) at 1mM final concentration, and 100 µL of the aqueous phase of the centrifuged culture medium. Samples were incubated at 30°C for 20 min and the reaction was stopped by the addition of100 µL of 5% trichloracetic acid (v/v in water).

Activity measurements were made spectrophotometrically at a wavelength of 420 nm by triplicate. The formation of the cationic radical was detected by measuring the increase in absorbance at 420 nm (ε_{420} = 36 000/M cm). One unit of enzymatic activity (U) is defined as the amount of enzyme catalyzing the oxidation of 1 µmol of ABTS in a 100 µL reaction at 30°C in 1 min.

The concentration of protein in the culture medium was determined by the method of Bradford with the BioRad Protein Assay kit (BioRad, USA) using bovine serum albumin as a standard. In order to evaluate the effect of CuSO4 (150 µM) and ethanol (10% v/v) on the extracellular laccase activity, the results were analyzed by one-way analysis of variance (ANOVA) and Tukey tests ($p<0.01$), using StatistiXL ver. 1.8.

RESULTS

Isolation of strains and tannic acid oxidation

The two isolated strains showed a white cottony mycelium growth in PDA medium, which in the presence of 0.5 g/L tannic acid produced a brown coloration of the medium, indicating the oxidation of the added substrate and suggest extracellular laccase activity (Figure 1).

Molecular genetic identification of isolated strains

The results of the Blastn search showed that the ITS

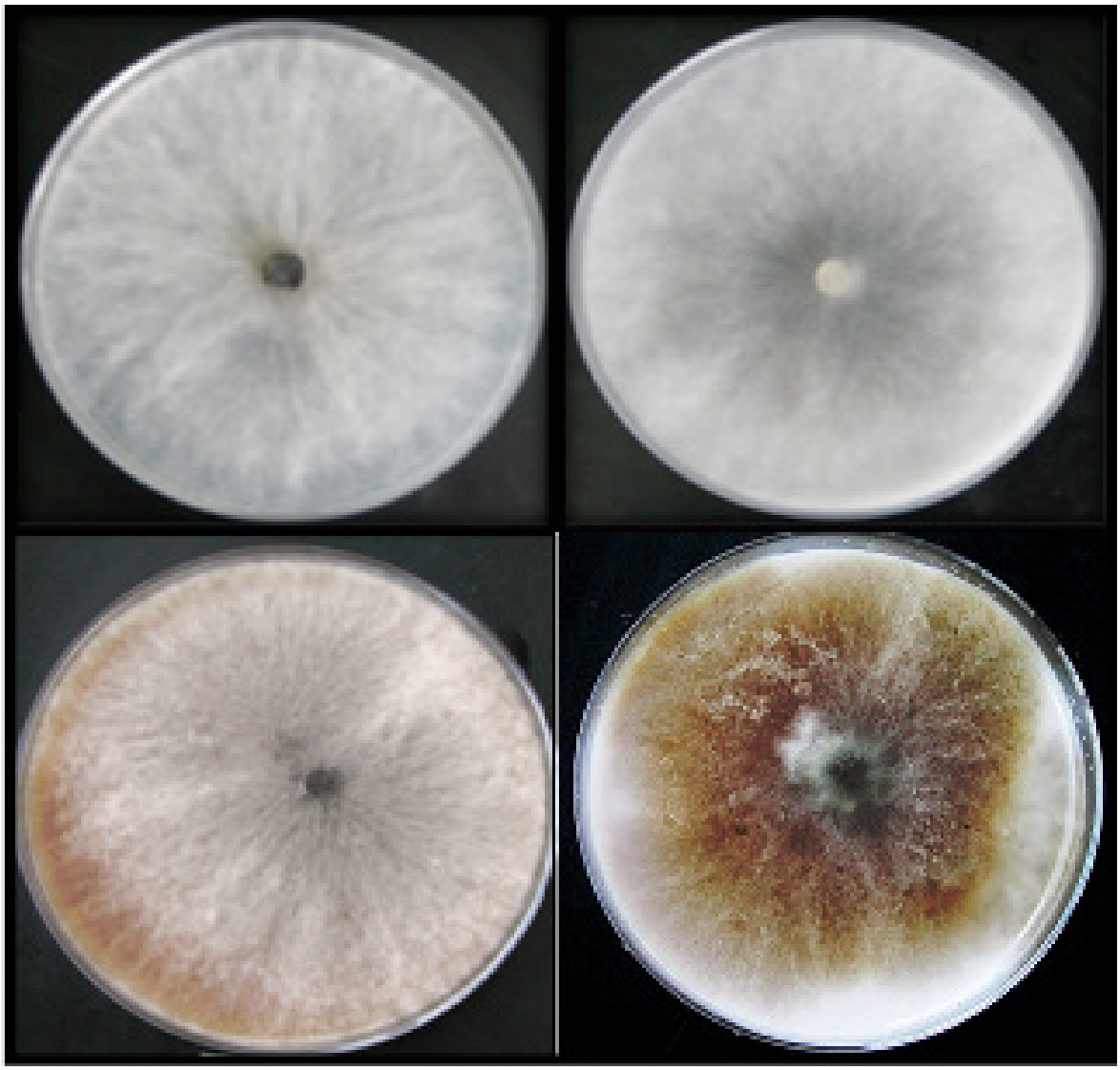

Figure 1. Colony growth of strains BM-1 (left) and BG-2 (right) in PDA medium after 48 h of incubation at 28°C in the absence (above) and presence (below) of tannic acid (0.5 g/L). Notice the brown coloration generated by the oxidation generated by the presence of tannic acid.

region of both isolated strains had a maximum similarity with that of several strains of *Botryosphaeria rhodina* and of its anamorphs in the genus *Lasiodiplodia* (data not shown). The lower similarity value was of 96% between *L. gonubiensis* and strain BG-2 and of 97% between *Lasiodiplodia rubropurpurea*, a strain of *L. theobromae*, a strain of *B. rhodina* and BM-1. The generated Neighbor-Joining tree (Figure 2) supports the placement of BG-2 and BM-1 within *B. rhodina/L. theobromae* complex.

Growth in liquid medium and production of extracellular protein of isolated strains

Growth kinetics shows that strain BM-1 reaches it maximal biomass production (35.40 ± 2.11 mg) during the second day of incubation and remains in stationary state without significant changes throughout the incubation period (Figure 3A). Strain BG-2 reaches maximum biomass production (31.10 ± 1.81 mg) at the third day of incubation and, as BM-1, presents no significant loss throughout the incubation period (Figure 3A). The addition to the medium of ethanol and copper sulfate had no significant effects on the growth kinetics of both strains (data not shown). The kinetics of production of extracellular protein of both strains in basal conditions shows an increment along the whole incubation period, towards the tenth day reaching values of 4.39 ± 0.35 µg/mL for BM-1 and of 3.61 ± 0.28 µg/mL for BG-2 (Figure 3A). In both strains, a significant increase in

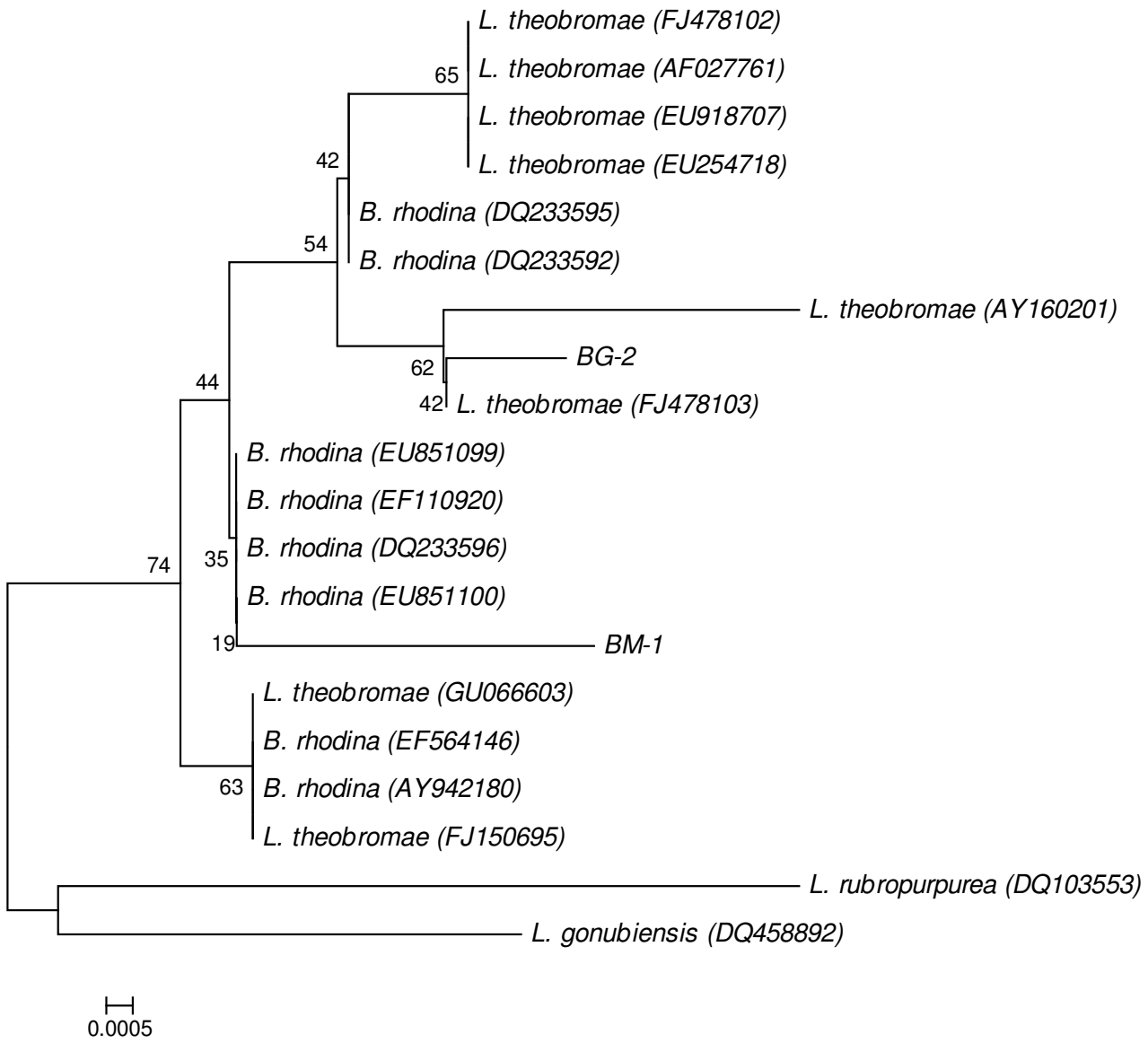

Figure 2. Neighbor-Joining tree of the ITS region of strains BG-2 and BM1 and those with higher similarity from the Genbank selected from the results of a Blastn search. Shown beside each node is the bootstrap value generated with 500 iterations. Genbank accession numbers of sequences used in the analysis are shown between parentheses. Main clusters in tree are denoted by the Romanic numerals. The tree was generated using the MEGA 4 software.

extracellular protein production is observed after the addition to the medium of either 10% ethanol or 150 µM $CuSO_4$ (Figure 3A), although the increase is larger in strain BM-1. In the latter strain, extracellular protein production was of 7.63 ± 0.60 µg/mL in the presence of ethanol and of 8.49 ± 0.37 µg/mL in the presence of copper, which in relation with the control culture represents a rise of 73 and 93%, respectively. In the case of strain BG-2, extracellular protein production was of only 4.72 ± 0.79 µg/mL with added ethanol and of 4.96 ± 0.32 µg/mL with the addition of copper, which is equivalent to less than 40% increase in comparison with the control culture.

Determination of extracellular laccase activity

The results of enzymatic activity assays of extracellular laccase showed low or undetectable basal activity in both strains throughout the incubation period. However, addition of Cu^{+2} or ethanol induced enzymatic activity in both strains (Figure 3B). The maximum basal activity displayed by strain BM-1 was of 0.379 ± 0.042 U/mL and of 0.312 ± 0.130 U/mL for strain BG-2, which in both cases was reached by the seventh day of incubation. For stain BM-1, the increase in extracellular laccase activity by the addition of ethanol or copper was approximately ten times that of the control culture, being of 3.639 ±

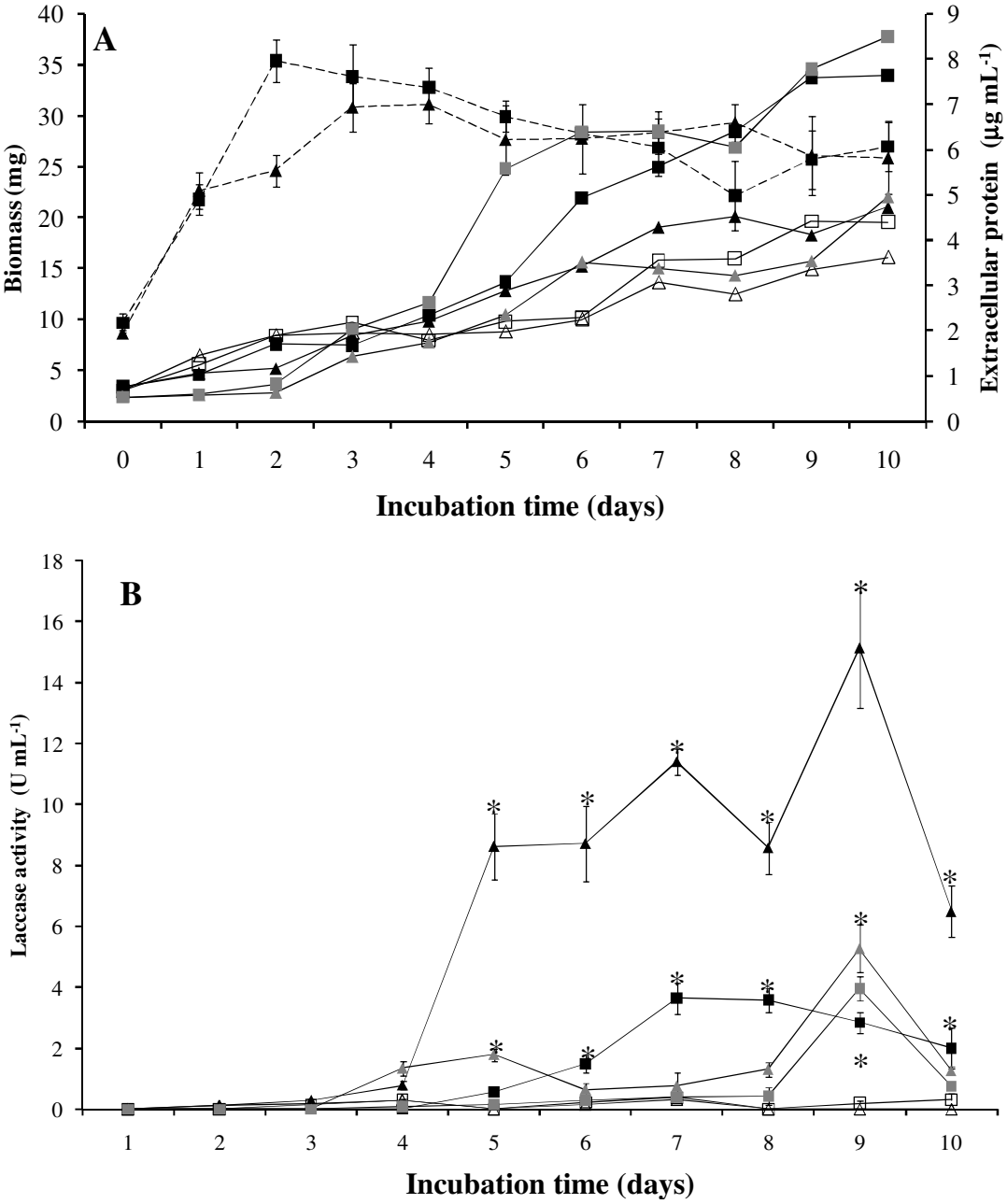

Figure 3. Line graphs of results of growth kinetics, extracellular protein production (Panel A) and extracellular laccase activity (Panel B) of strains BM-1 (squares) and BG-2 (triangles). Both panels show data of cultures supplemented with 10% ethanol (dark symbols), 150 µM $CuSO_4$ (grey symbols), and the control (empty symbols). Panel A: Growth kinetics (dashed lines) of control cultures and produced extracellular protein (straight lines) of control and induced cultures. Growth kinetics of induced cultures are not shown, however, these are not significantly different from that in the control cultures. For visual clarity standard deviations of extracellular protein production are omitted, which are not larger than 10% of the mean value. Panel B: Extracellular laccase activity in cultures supplemented with 150 µM $CuSO_4$ (dashed line) and 10% ethanol (solid line). All values shown in graphs represent the average of three independent experiments made in triplicate. Activity values in induced cultures which are significantly higher (AMOVA, $p < 0.01$) than its respective point in the control cultures are marked with an asterisk.

0.506 U/mL for the former and of 3.964 ± 0.385 U/mL for the latter inducer. Such maximal activity was reached by the seventh day of incubation with added ethanol and on the ninth day when copper was added to the medium

(Figure 3B). Extracellular laccase activity shows a significantly larger increase with the addition of ethanol than with supplementation of Cu^{+2}, although in both cases the observed kinetics is complex showing several activity peaks. In the case of copper addition, a first peak of 1.784 ± 0.199 U/mL is observed at five days of incubation and a maximum activity peak of 5.270 ± 0.793 U/mL at the ninth day (Figure 3B). For cultures with ethanol, the first peak of enzymatic activity of 8.713 ± 1.237 U/mL is observed at five days of incubation followed by a second increase to 11.397 ± 0.440 U/mL by day seven, the maximum activity of 15.142 ± 1.975 U/mL occurring by the ninth day. In these cultures, extracellular laccase activity remains relatively high until the last day of evaluation at a value of 6.483 ± 0.845 U/mL (Figure 3B).

DISCUSSION

Two strains of wild fungi were isolated from rotting stemwood of the tree *Ipomoea murucoides*, a species of tropical tree endemic to Mexico and Guatemala (Rico, 1985) that is widely distributed in Mexico and which is able to colonize areas disturbed by agriculture. The stem of this tree species constitutes a new lignin substrate from which there were not previously isolated ligninolytic fungi.

Molecular genetic analysis of the ITS region of the isolated strains clearly identified them with *Lasiodiplodia theobromae*, an anamorph of *Botryosphaeria rhodina*. The usefulness of the ITS region for differentiating between species of the genus *Botryosphaeria* and its anamorphs had previously been demonstrated (Denman et al., 2000; Zhou and Stanosz, 2001), a fact which strengthens the taxonomic identity of the strains isolated in the present work. The species of the genus *Botryosphaeria* cause several disease symptoms in a large number of woody plants in combination with stress-inducing environmental conditions (Schoeneweiss, 1981; Gilbert and Hubbell, 1996). The taxonomy of the genus has been traditionally based on the morphology of anamorphic states, which are the most commonly found in nature. However, many of the morphological features used for taxonomic delimitation are shared between apparently unrelated taxa. For example, some anamorphs of related genera within the family Botryosphaeraceae present conidia with similar morphology and size. Comparison of DNA sequences has recently been used for delimiting species of the genus *Botryosphaeria* (Burgess et al., 2006; De Wet et al., 2008). In solid media cultures, the presence of conidia was never observed in the incubation conditions used by us, but since the induction of propagation structures in the isolates was beyond the scope of the present work, it is possible that conidia may have appeared under different culture conditions.

Both isolated strains showed a strong oxidation reaction in the presence of tannic acid, which is an indicator of the presence of extracellular phenol oxidases (Thompson and Cannon, 1984). Tannic acid is a polyphenolic compound in the group of hydrolysable tannins that can induce the production of extracellular laccases in fungi (Carbajo et al., 2002; Chung et al., 2008). Strains were studied in liquid medium cultures, in order to assess biomass and protein production as well as extracellular laccase activity. Both strains grew rapidly in the liquid medium used and produced similar levels of extracellular protein in conditions of basal incubation. The addition of ethanol and of copper significantly increased the production of extracellular protein in both strains, although such increment was larger in strain BM-1.

The extracellular laccase activity was initially reported in strains from the wild isolate MAMB-05 of *Botryosphaeria rhodina* after induction with veratryl alcohol, using as a substrate the polymeric colorant Poly R-479 (Barbosa et al., 1996). It has been documented that MAMB-05 produces two constitutive extracellular laccases, PPO-I and PPO-II, which in the presence of veratryl alcohol increase their activity up to 100 and 25 times, respectively (Vasconcelos et al., 2000; Dekker and Barbosa, 2001). The latter study showed that both laccases increased their activity by 4 to 5 times in aerated cultures in relation to non-aerated cultures. The inducing effect of extracellular laccases by veratryl alcohol has recently been reported in six wild isolates of *B. rhodina* from Brazil (Saldanha et al., 2007). Evaluations have also been made in MAMB-05 isolate of the effects of carbon (Dekker et al., 2001; Alves da Cunha et al., 2003) and nitrogen (Dekker et al., 2007) sources on extracellular laccase activity, both constitutive and induced by veratryl alcohol.

The comparison of the activity of extracellular laccase in the *B. rhodina* isolate MAMB-05 and in the herein studied isolates must be made with caution, given that in the former case the measurements of activity were made in Vogel defined medium and in the latter, in complete PDB medium supplemented with yeast extract. In our cultures, 2% glucose was used while the initial studies of MAMB-05 were made in cultures with 1% glucose (Dekker and Barbosa, 2001). Despite these differences in culture medium it is interesting to contrast our results with those previously reported for isolate MAMB-05. Maximum activities of extracellular laccase in non-induced cultures of BM-1 and BG-2 are higher than those of the PPO-II laccase, but lower than those described for the PPO-I laccase reported for the isolate MAMB-05 in cultures with 1% glucose (Dekker and Barbosa, 2001). The kinetics of enzymatic activity in basal culture observed by us, differs from that reported for the MAMB-5 isolate; while the maximum activity for the isolates studied by us occurs on day seven of incubation, in the latter isolate it occurs on the ninth day for PPO-I and in the fifth for PPO-II. These data show the existing

physiological variation in the BM-1 and BG-2 strains and in the MAMB-05 isolate.

In Ascomycetes, the induction of extracellular laccase activity by copper ions was reported in *Podospora anserine* (Fernández-Larrea and Stahl, 1996), *Gaeumannomyces gramminis* var. *tritici* (Edens et al., 1999; Litvintseva and Henson, 2002), and more recently in *Botryosphaeria rhodina* (Dekker et al., 2007). Based on those reports, the effect of copper on extracellular laccase activity was assayed in the present work. The addition of copper caused an increase in maximum extracellular laccase activity of approximately eleven times in the BM-1 strain, and of seventeen times in the BG-2 strain, compared to the basal culture. These increases are very near to those previously reported for *B. rhodina*, in which the addition of 160 µM of copper sulfate (a concentration that is very similar to that used by us) increased by 15 times the activity of extracellular laccase (Dekker et al., 2007). Copper is a cofactor of fungal laccases (Hoshida et al., 2005) and the ion has been reported to be a transcriptional regulator of laccase activity (Collins and Dobson, 1997; Litvintseva and Henson, 2002). Metal response elements (MRE) have been reported to be present in the promoter regions of the laccase genes (Litvintseva and Henson, 2002). The possibility of the presence of MRE in strains BM-1 and BG-2 or in other strains of *Botryosphaeria* and its anamorphs in *Lasidioplodia* would be an interesting subject of future studies.

The induction in fungi of extracellular laccase by ethanol was reported for the first time in *Trametes versicolor* (Lee et al., 1999). In fact, several primary and secondary alcohols were tested in this species, of which only methanol and ethanol showed a significant induction of laccase in aqueous medium. In the case of *Rhizoctonia solani*, both isopropanol and ethanol significantly increased the activity of extracellular laccase in aqueous medium (Crowe and Olsson, 2001). Another species in which ethanol induced an increase in extracellular laccase activity is *Pycnoporus cinnabarinus* (Lomascolo et al., 2003). Dekker et al. (2001) comment that ethanol stimulated the production of extracellular laccase in the MAMB-05 isolate of *B. rhodina*, but the authors did not provide any additional data allowing for comparison with the results here presented. With the addition of ethanol to the culture medium, the maximum activity of laccase was increased by approximately ten times in strain BM-1 and by 36.5 times in isolate BG-2, in relation with basal incubation conditions. Such increment was of 11.37 times compared to the control in *T. versicolor* after the addition of 20 g/L of ethanol (Lee et al., 1999), while in *P. cinnabarinus* a 155 times increment of the enzymatic activity relative to the control culture was registered when adding 35 g/L of ethanol (Lomascolo et al., 2003). While the addition to the culture medium of ethanol induces a higher increase in production of extracellular protein in strain BM-1 than the same in strain BG-2, the induced activity of extracellular laccase is larger in the latter strain, which suggests that strain BG-2 produces more extracellular laccase than strain BM-1. The kinetics extracellular laccase activity in the presence of ethanol of strain BG-2 suggests the possibility that it synthesizes more than one isozyme, given that at least to peaks of activity are observed during the incubation period. As mentioned above, the presence of two isozymes of extracellular laccase has been reported in *B. rhodina* (Dekker and Barbosa, 2001). It has been proposed that ethanol may indirectly induce the synthesis of laccases through the oxidative stress in cellular membranes brought about by the alcohol, which in some fungi induces the expression of laccases and peroxidases (Crowe and Olsson, 2001); however, it is not known if the molecular mechanism of induction by ethanol is similar to that of the induction by oxidative stress. In *T. versicolor*, ethanol has been observed to induce the synthesis of laccases at the same time it inhibits the synthesis of melanin (Lee et al., 1999), because of which it has been postulated that the monomers of melanin pigments (which are aromatic compounds) that accumulate due to the effect of ethanol are responsible for the induction of laccases, an effect that has been sufficiently documented for other aromatic structures (Lee et al., 1999); nevertheless, the molecular mechanisms by which such induction would occur have not been described.

The results of the present study showed that the obtained isolates belong to *Botryosphaeria rhodina*. Regarding extracellular laccase production, the two studied isolates display significant differences. Also, both isolates showed a pattern of extracellular laccase production different from that previously observed in the isolate MAMB-05 of *B. rhodina*. The present study contributes to emphasize the relevance of continuing the isolation and characterization of wild fungi strains able of producing extracellular laccases on different lignocellulosic substrates and belonging to taxonomic groups that have been little studied in that regard.

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