Indian Journal of Biotechnology
Vol. 18, January 2019, pp 42-51

Laccase gene silencing negatively effects growth and development in *Pleurotus ostreatus*

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Received 12 December 2018; revised 27 December 2018; accepted 30 December 2018

In this study we describe the effects of *Pleurotus ostreatus* transformants that have been silenced for laccase genes. Using the RNAi strategy, transformants with different levels of phenotypic alteration were obtained with respect to their oxidation capacity of 2,6-dimethoxyphenol (DMP). The analysis of laccase activity on DMP allowed us to select transformants with severe, medium and light phenotypic alteration in comparison to the PoB strain. The measurements of the average growth rate of the transformants with severe phenotypic alteration suggested that laccase expression could play a role in the vegetative growth of *P. ostreatus*. It was observed that both in solid and liquid cultures, PoB and the transformants express mRNA for lacc10, although the transformants with medium and severe phenotypic alteration present a decrease in intensity, especially in solid culture. This suggests that the product of this gene is responsible for the development of the mycelium and probably participates in the production of biomass in solid culture and also could be related to the decrease in the intensity of the constant isoenzyme observed in both culture systems.

Keywords: RNAi, laccase gene silencing, *Pleurotus ostreatus*, fungi, phenol oxidases, phenotype, physiology

Introduction

Pleurotus ostreatus is a saprotrophic fungus, in its natural environment this fungus grows on branches and trunks of fallen or decaying trees and even on the ground on rotten roots[1]. The fungus is an edible mushroom that is cultivated on various lignocellulosic

coffee pulp and other agro-industrial residues. It has been suggested that this is possible because this fungus produces an enzymatic complex that is responsible for the degradation of the lignin of these natural substrates. This enzymatic complex is primarily composed of lignin peroxidase, manganese peroxidase and laccase[2]. *P. ostreatus* has been converted into a fungus of biotechnological interest due to its ability to produce laccase enzymes, which have multiple applications at the biotechnological[3] and environmental levels[4]; thus, at present, there are many studies regarding these enzymes. The role played by the laccases in the different organisms in which they have been found has been investigated, i.e., the role of bacteria[7]. In the case of fungi, it has been observed that the laccases have a variety of functions. In ascomycetes, it has been suggested that laccases participate in the biosynthesis of melanin in conidia[8], in pigmentation of cleistothecial primordia[9] and pathogenesis[10]. Furthermore, in basidiomycetes, it has been suggested that the laccases participate in mycelial development in *Agaricus bisporus*[11], in the development of primordia and the synthesis of melanin in the fruit body of *Lentinula edodes* and even in the basidiome morphogenesis[12-13]. In the white rot basidiomycete *Pycnoporus cinnabarinus*, the production of a single

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laccase isozyme and traces of a peroxidase that is neither LiP nor MnP was identified. Therefore, the degradation of lignin by this fungus is attributed almost exclusively to laccases[14]. It was also reported that the characteristic red pigment produced by *P. cinnabarinus* is formed by the oxidation of the 3-hydroxyanthranilic acid (3-HAA) precursor to cinnabaric acid during the formation of the fruiting body, in a reaction catalyzed by laccase[15]. Generally, laccases have been attributed a significant role in the degradation of lignin in white rot fungi[16]. However, the most important fungus that degrades lignin, *Phanerochaete chrysosporium*, does not produce any typical laccases but rather produces an enzyme with ferroxidase activity[17].

Despite these findings, the physiological function of laccases in *Pleurotus* has been scarcely studied; several authors have suggested that these proteins participate in mycelial development[18], sporulation[19], and in fruiting body development[20]. Conversely, a high percentage of altered transcriptional responses in laccase genes has been observed during its culture in the presence of an extract of wheat straw, which suggests that the laccases are sensitive to phenolic compounds and sugars present in the inducer extract[21]. In addition, this could indicate a possible function of laccases in the assimilation of lignin as a complex carbon source in its natural habitat. However, almost all of the *in vitro* studies intended to produce laccase enzymes have used glucose as a carbon source in the cultures, in addition to variation of other factors, which is not in keeping with the assessments made previously regarding their role in the assimilation of a complex carbon source. This suggests that laccase's physiological participation could be different.

Pezzella[22] performed transcriptional analysis of nine laccase genes in *P. ostreatus* to attempt to unravel the physiological role played by the different laccase isoenzymes produced in this fungus. These researcher's results showed a complex picture of transcriptional responses exhibited by the laccase genes analyzed; therefore, the authors suggested that the genetic manipulation of fungal systems to silence laccases would be useful to further disentangle the enigma concerning the function of laccase enzymes.

One strategy that is considered to be a powerful tool to identify the function of genes in a wide variety of eukaryotic organisms, including fungi, is employing a mechanism of genetic regulation through the use of interfering RNA (RNAi). The RNAi is a mechanism of post-transcriptional gene silencing that result in degradation of the produced RNA messenger, attenuating the expression of the target gene. Through silencing of the genes coding for the enzymes of interest, the role of these enzymes in the physiology of the organisms can be verified according to the hypothesis that "the function of a gene can be directly tested in experiments in which a gain or loss of activity can be predicted by the presence or absence of a functional gene"[23]. Among the main advantages of using RNAi in functional genetics research is that it can be applied to the analysis of the function of multigenic families, such as the genes encoding laccases in *P. ostreatus*[24]. In a study conducted by Salame *et al* (2010), the silencing of the sequence target *mnp3* in *P. ostreatus*, induced a strong silencing of *mnp3* and *mnp9* and also reduced the transcription of *mnp4* without substantially affecting the expression of other members of the family. RNAi also allows for the analysis of genes whose elimination is lethal, since it attenuates the expression of specific genes at distinct levels, which can maintain organism viability[25]. This feature can be used to select transformants with phenotypic variations, the analysis of which can provide information related to the function of the target gene[26].

In this work, we used the strategy of RNAi to obtain transformants with distinct levels of attenuation of laccase genes expression. Next, we evaluated the phenotypes obtained with respect to their laccase activity and their effect on mycelial growth to try to corroborate the involvement of the laccases in the early stages of fungus development, and the effect on laccase activity on the development of the transformants when they are grown in solid and in liquid media.

Materials and Methods

Strain

P. ostreatus strain PoB was obtained from the institutional collection of edible mushrooms of the Autonomous University of Chapingo, Mexico. The strain was received as dikaryotic mycelium and it is wild-type. The conservation of the *P. ostreatus* strain was performed on malt-extract agar medium (BD Bioxon®) at 25°C in Petri dishes of 90 mm diameter for 10 days and then it was stored at 4°C.

Plasmid

The plasmid pRNAi-LAC[27] was developed from the plasmid pGpdPki-RNAi[28]. The plasmid pRNAi-LAC contains the promoter *gdh* of the *Aspergillus*

nidulans gene, the terminator of the *cyc1* of the *Saccharomyces cerevisiae* gene and the gene for resistance to phleomycin (*ble*) is used as the selection marker. In addition, this vector contains the genes promoters *gpd* and *pki* of *A. nidulans* and *A. niger*, respectively, placed in opposite directions and facing each other such that transcription from each promoter generates a double RNA strand complementary to a fragment of the gene inserted between them. For this purpose, the plasmid has two *Nco*I restriction sites, into which was inserted a DNA fragment of 317 bp (5'-ATGTCGTGGCCCCTCAAAATGCGGGGCTC GCCCACTCCCGATAGACACTCATCAATGGCAT AGGTCGCTACGCTGGGGGTCCTACTTCCCCTC TGGCCGTCATCAACGTCGAAAGCGGCAAGCG ATATCGTTTCCGACTCATCTCCATGTCTTGCG ACCCCAATTACACGTTCTCGATCGACGGTCAC ACTTTGCCGGTTATTGAAGCAGATGCAGTCAA TATTGTGCCCCTTGACGGTATGTTTCATATAC TGACAACACAAACACATTCTGAATCATCTTGA TGTAGTGGATAGTAT-3'), the analysis of which with BLAST was found to correspond to 77% identity to the *pox4* gene laccase exon 10 between position 1491-1808 (Accession FM202670) of *P. ostreatus* ATCC no. MYA-2306 (Fig. 1 and 2).

Culture Media

The culture medium used for the fungal transformation consisted of five Erlenmeyer flasks each with 25 mL of YMG medium[29] (per litre: 4 g yeast extract, 10 g malt extract and 4 g glucose) for the propagation of approximately 0.05 g of mycelium, previously homogenized with an electric mixer (Aerolatte®) for 5 min. The cultures were incubated at 25°C at 120 rpm for 60 hours. This experiment was performed in triplicate. The evaluation and screening of transformants for laccase activity was carried out in 60 mm diameter Petri dishes with 10 mL of YMG agar containing 0.5 M 2,6-dimethoxyphenol (Sigma Aldrich®) (YMG-agar-DMP). This experiment was performed in triplicate.

For the evaluation of the silencing of laccase genes, the culture was carried out in both solid and liquid cultures. The solid culture was carried out in 90 mm diameter Petri dishes containing 25 mL of YMG agar. For the liquid culture, Erlenmeyer flasks were used with 25 mL of YMG. The cultures were incubated at 25°C for 7 days in darkness. Both experiments were performed in sextuplicate. Three replicates were used to obtain the total RNA and the other three replicates were used to estimate the produced biomass.

The estimation of the growth rate average (GRA) was carried out in 90 mm diameter Petri dishes containing 25 mL of YMG agar. This experiment was performed in triplicate. The inoculum for all culture conditions was one disk (8 mm diameter) of mycelium taken from the edge of a freshly grown colony, positioned in the centre of the Petri dish

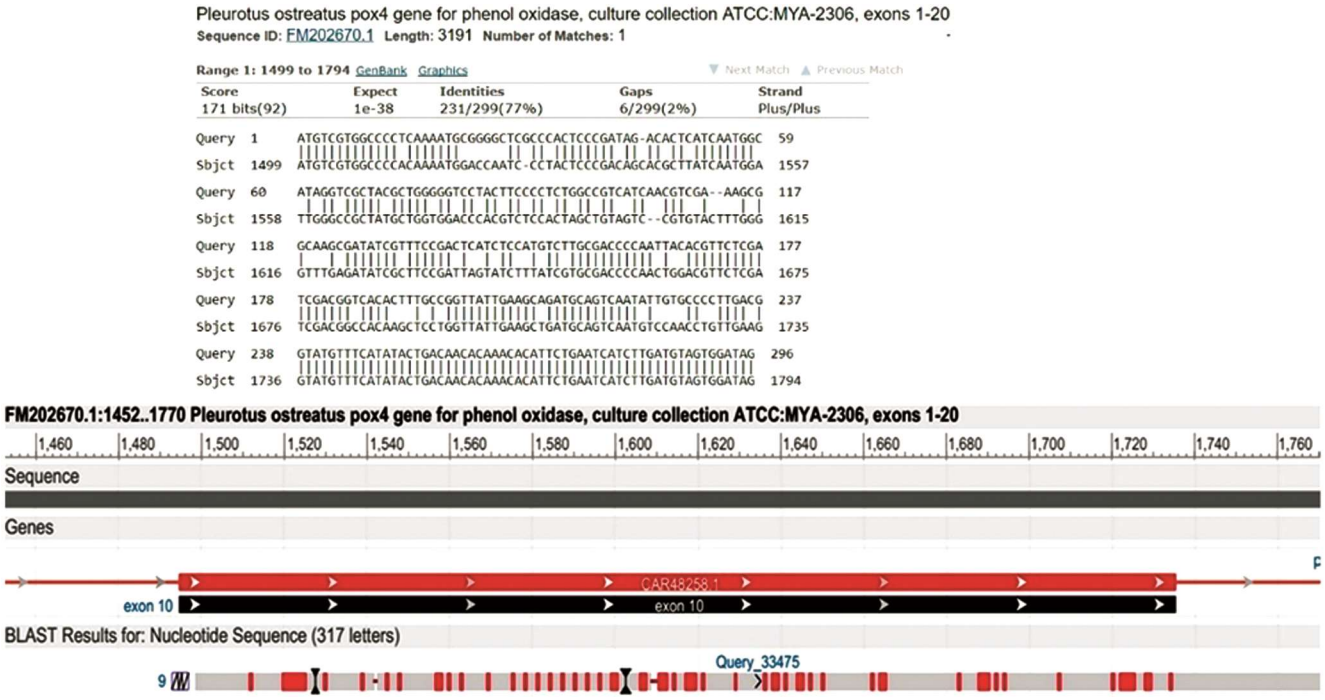


Fig. 1 — Alignment of the DNA sequence used (317 bp) in the sequence databases.

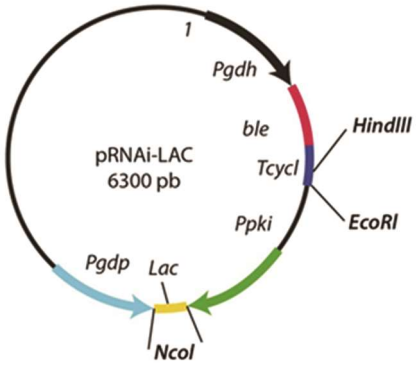


Fig. 2 — Plasmid pRNAi-LAC.

and randomly positioned when an Erlenmeyer flask was used.

Fungal Transformation

The mycelium cultivated in the YMG medium was made competent by treatment with lytic enzymes and the process is described below: The mycelium was centrifuged and resuspended in the YMG medium after adding 0.6 M of sucrose (Meyer® reagents) as osmotic stabilizer, and lytic enzymes of *Trichoderma harzianum* (with β-glucanase, cellulase, protease, and chitinase) (Sigma Aldrich®) were added at a concentration of 10 mg/mL and allowed to incubate for 1.5–2.0 h at 32°C with gentle shaking.

This process weakens the cell wall facilitating the transformation by electroporation. The treated mycelium was washed three times by centrifugation-resuspension in milli-Q water and were eventually resuspended in milli-Q water. Immediately thereafter electroporation was conducted using the conditions described by Peng[30], except that in this study 2.2–2.8 kV/cm field strength and 100–500 Ohms resistance was used, in 0.2 cm electrode cuvettes, using a Gene PulserXcell™ Electroporation Systems (Bio-Rad®).

To obtain transformants, 40 µL of mycelial suspension (protoplast 1×10^7) was mixed with 300 ng of the plasmid pRNAi-LAC and as a control 40 µL of mycelial suspension (protoplast 1×10^7) was mixed with 300 ng of the plasmid without the insert (PoB2) followed by the electroporation. Additionally, protoplasts without plasmid were also electroporated and used as a control for the effect of the electroporation (PoB1). In addition, protoplasts not subjected to electroporation were used as a control for the digestion with lytic enzymes (PoBsine). This experiment was carried out in triplicate. Immediately after electroporation, 800 µl of 0.6 M sucrose, as an osmotic stabilizer, was added to every replicate of the mycelial suspension, and the mixtures were placed on ice. Subsequently, the regeneration of the mycelium in Erlenmeyer flasks with YMG medium at 25°C at 120 rpm for 4–7 days was performed. The regenerated strains were isolated and were individually grown on plates with YMG-agar medium and then were used in the evaluation.

Evaluation and Screening of Transformants

A phenotypic analysis was performed in triplicate measuring the oxidation halo of DMP produced on the solid medium by the pRNAi-LAC transformants and the parental strain followed by the selection of transformants with different levels of DMP oxidation. The genotypic analysis was performed with confirmation of the integration of the constructs into the selected genome strains by PCR using the primers Pgdp and Ppki, followed by an analysis of the obtained sequences to confirm the presence of the plasmid pRNAi-LAC.

A culture test was performed to observe the oxidation halo and determine the number of isoenzymes present in the parental strain (PoB), the control of the transformation (PoB2), the control of the electroporation (PoB1) and of the control of digestion (PoBsine). The following experiments were carried out using only the selected transformants and the parental strain PoB.

Evaluation of Laccase Genes Expressed in Solid and Liquid Cultures

Total RNA extraction, reverse transcription and PCR. The biomass obtained in both cultures was processed to obtain total RNA, using a RNeasy plant mini kit (QIAGEN®) according to the manufacturer's protocol. The genomic DNA was removed from the RNA samples using DNAse I (Thermo Scientific®). The integrity of the RNA samples was verified by visualization on ethidium bromide stained agarose gels and their concentration was determined spectrophotometrically using the smartspect plus spectrophotometer (Bio-Rad®). For the synthesis of the first strand complementary DNA (cDNA) the revert aid H minus first strand cDNA synthesis kit (Thermo Scientific®) was used with 3 µg of total RNA as template. To perform the PCR amplification, 2 µl of the product of the cDNA synthesis was used as a template for PCR with 200 µM of dNTPs mix, 2 µM $MgCl_2$, and 0.1 U of *Taq* DNA polymerase in KCl buffer (Thermo Scientific®) and 0.4 µM of each pair of oligonucleotides in a 50 µl total volume. The pairs of primers that we used to evaluate the expression of the laccase genes are those corresponding to the

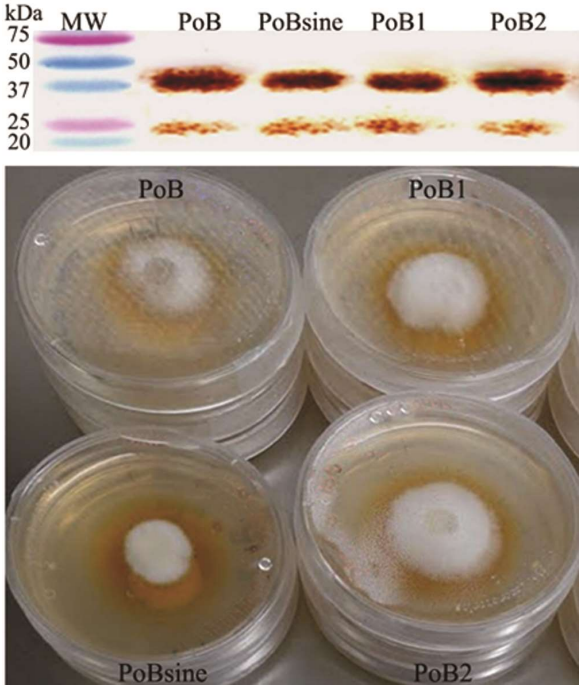


Fig 3 — Isoenzymes and oxidation halo presented by the controls: (PoB) parental strain, (PoBsine) control of the digestion with lytic enzymes, (PoB1) control of the effect of the electroporation without plasmid, (PoB2) control of the transformation with the plasmid without the insert.

lacc1, *lacc2*, *lacc3*, *lacc4*, *lacc6*, *lacc7*, *lacc8*, *lacc10*, *lacc12* and *actin* genes reported by Pezzella[22].

The PCR was performed on a T100 thermal cycler (BioRad®) with the following conditions for the pairs of laccase primers: 95°C for 4 min and 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min followed by 72°C for 7 min; the *actin* gene (constitutive expression gene) was used as control, the same conditions were used, but instead of using 55°C for the ringing step, 58°C was used for 1 min. The result of the amplifications was visualized on agarose gels using 1X TAE, stained in a solution of ethidium bromide with a final concentration of 0.5 µg/L. The DNA in the gels was visualized in a DigiDoc-it photo-documenter (UVP®). GeneRuler™ 100 bp DNA Ladder (Thermo Scientific®) was used as molecular weight marker. The normalized intensity of the amplicons obtained was obtained using the ImageJ program, which is a public domain digital image processing program programmed in Java developed at the National Institutes of Health. With the program, data of relative intensity are obtained and by dividing the relative intensity of each mRNA and each strain between the respective relative intensity of actin, the value of the normalized intensity was obtained.

Estimating the Average Growth Rate

To estimate the average growth rate (AGR) of the transformants with respect to the control strain PoB in the solid culture, cultures were carried out in 90 mm diameter Petri dishes with YMG agar. Every 24 h and during a period of 7 days, the diameter of each colony was measured vertically and horizontally (longest and shortest diameter) to calculate an average diameter, using a digital vernier. This experiment was performed in triplicate. The radial growth of each strain was fitted to a linear equation. The value of the ordinate at the origin (b) was established in the diameter of the fragment inoculated (8 mm) and where the GRA represents the slope (m) of the line.

Obtaining Enzymatic Extracts and Biomass in the Solid and Liquid Cultures

To obtain the enzymatic extract (EE) from the solid culture, the procedure was as follows: the mycelium was removed with the aid of a platinum handle, the resulting medium (mycelium free) was cut into approximately 1×1 cm^2 cubes and placed in a 125 mL flask, and 25 mL of 0.5 M pH 6.5 phosphate buffer was added to the flask. To obtain the EE of the liquid culture, this was filtered, retaining the biomass produced with the aid of a Buchner flask. The resulting enzymatic extracts were used for both obtaining the number of laccase isoenzymes and the laccase activity. The obtained biomass was quantified as dry weight (oven drying at 40°C, and subsequently recording its weight).

Obtaining the Number of Laccase Isoenzymes by Zymography

To observe the number of isoenzymes present produced by the transformants strains and PoB, their activity was detected *in vitro* using 10 µg of protein from each EE in zymograms based on the modified SDS-PAGE technique of Laemmli[31]. The separating gel contained 12% acrylamide and 2.7% bis-acrylamide. The packaging gel contained 4% acrylamide and 2.7% bis-acrylamide. Samples of enzyme extracts were run on 0.75 mm thick gels at 150 V in a mini-PROTEAN tetra cell (BioRad®) electrophoresis system for 1–2 h. After the run, the gels were washed with distilled and deionized water for 1–1.5 h, with water change every 15 min to remove the SDS and re-nature the proteins. Finally, the gels were incubated for 12 h at room temperature in 50 mL of 2 mM DMP in 0.1 M pH 6.5 phosphate buffer.

Laccase Assay

The laccase enzymatic activity present in the EEs was determined with the following reaction mixture: 800 µl of 2 mM DMP in 0.1 M pH 6.5 phosphate buffer and 200 µl EE. The product was measured following a reaction kinetics for 1 min at 39°C at 468 nm on a smartspect plus spectrophotometer (BioRad®). The enzymatic activity was expressed as international units (IU), where 1 IU is defined as the amount of enzyme that catalyses the transformation of 1 µM of the substrate into product per minute. To obtain the specific activity, the total protein produced was measured by the Bradford method[32].

Statistical Analysis

All growth rate averages, biomass and specific laccase activity measurements were analyzed using a one-way ANOVA test ($\alpha = 0.05$) to determine the statistical significance of the differences. Next, differences between the transformants and the PoB strain were evaluated using the Dunnet's test ($P \leq 0.05$). For this analysis, the XLSTAT Excel complement was used.

Results and Discussion

Fungal Transformation

Twenty-seven pRNAi-LAC transformants were obtained. The analysis of laccase activity on DMP allowed us to select transformants with severe, medium and light phenotypic alterations in comparison to the PoB strain. Most of the transformants presented an oxidation halo similar to that of the PoB strain, and among them, T7 was randomly chosen and considered to be a transformant with a light phenotypic alteration. T21 was selected because it showed a minor oxidation halo compared with both the PoB strain and the T21 transformant and was considered as a transformant with a medium phenotypic alteration. T27 was the only transformant that produced a halo of light oxidation, and T26 was the only transformant that presented with almost no oxidation halo, and both were considered to be transformants with severe phenotypic alteration. In the following experiments, we used the PoB strain as the reference. The oxidation halos produced after 48 hours by the PoB strain and the selected transformants selected are shown in Figure 3.

Evaluation of the Silencing of Laccase Genes

From the analysis of laccase expression, we obtained RT-PCR products with the laccase gene primers mentioned before with amplicons between approximately 50 and 100 bp expected. In Figure 4 shows the amplicons obtained for the five strains and the nine primers as well as the amplicons obtained for actin. The PoB mRNA had a higher intensity of expression for the *lacc1*, *lacc2*, *lacc4*, *lacc8*, *lacc10*, and *lacc12* genes in solid culture (Fig. 4a). The transformants expressed the mRNA for the nine primer pairs tested in the solid culture, although the mRNA intensity of the lacc7 gene was very low in all strains, except for the intensity shown for T27. The expression of the mRNA for the genes *lacc1*, *lacc3*, *lacc4* and *lacc6*, with respect to the control PoB, does not present a pattern that suggests a decrease according to the level of involvement of the transformants. Except for T27, mRNA expression for the *lacc10* gene decreased significantly according to the degree of involvement of the transformants. Meanwhile, in liquid culture (Fig. 4b), the mRNA was expressed in PoB for almost all laccase genes with the exception of the *lacc7* gene, whose intensity is almost null. The mRNA expression for *lacc2*, *lacc8*, *lacc10* and *lacc12* was observed in the liquid culture for all strains, but in the cases of *lacc4* and *lacc6*, an important decrease in its expression was observed in the T21 and T26 transformants.

It was observed that both in solid and liquid cultures, PoB and the transformants express mRNA for *lacc10*, although T21, T26, and T27 present a decrease in intensity, especially in solid culture. The expression of mRNA for the *lacc2*, *lacc8*, and *lacc12* genes has the highest intensities for the PoB control and the transformants in both cultures, also, T27 expressed the nine mRNAs for laccase, although some less intense than others. This has been an unexpected behavior since T27 is one of the transformants with severe phenotypic involvement. It is probable that T27 is using a compensation mechanism induced by the silencing. In this sense, a reprogramming mechanism carried out by paralogous genes, which are considered to have a backup function, is proposed[33]. It has been found that the laccase genes belong to a multigenic family[34]. Thus, it makes sense that this production of mRNA corresponds to a natural response, although not all of

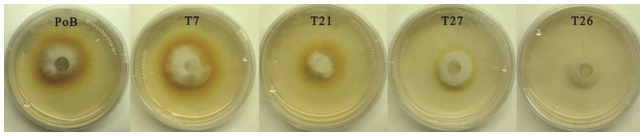
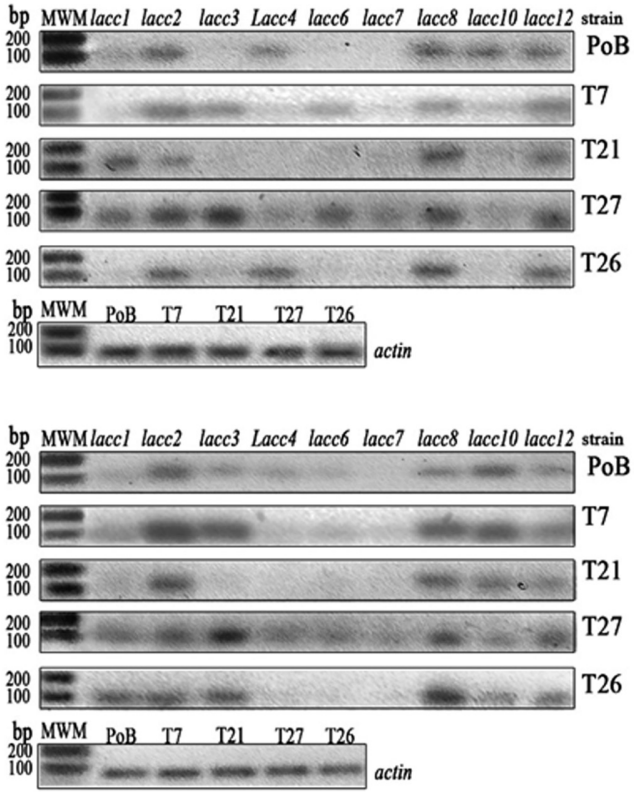


Fig. 3 — Laccase activity obtained by the parental strain PoB and by the transformants on DMP.



lacc1	lacc2	lacc3	lacc4	lacc6	lacc7	lacc8	lacc10	lacc12	strain
0.244	0.828	0.119	0.456	0.051	0.071	0.756	0.665	0.610	PoB
0.259	1.185	0.903	0.320	0.672	0.266	0.901	0.521	1.109	T7
0.526	0.346	0.076	0.030	0.127	0.128	0.563	0.109	0.385	T21
0.865	1.344	1.612	0.581	1.041	0.579	1.021	0.247	0.919	T27
0.147	0.592	0.309	0.599	0.249	0.082	0.744	0.095	0.551	T26

a)

lacc1	lacc2	lacc3	lacc4	lacc6	lacc7	lacc8	lacc10	lacc12	strain
0.252	0.757	0.315	0.281	0.161	0.033	0.374	0.603	0.296	PoB
0.635	1.384	1.310	0.427	0.561	0.393	1.443	1.196	0.695	T7
0.115	0.542	0.120	0.008	0.046	0.061	0.492	0.372	0.280	T21
1.035	1.089	1.537	0.953	0.770	0.523	0.992	0.516	0.950	T27
0.539	0.453	0.671	0.080	0.068	0.057	1.034	0.471	0.376	T26

b)

Fig. 4 — Right: mRNA of genes for laccases. Left: normalized intensity data. a) solid and b) liquid cultures.

the mRNAs produced are functional, since the T27 strain showed a severe phenotypic change in its ability of laccase production.

Average Growth Rate in Solid Culture

An analysis of the variance of the means of the AGR obtained was performed. The results indicated that there are significant differences between the AGR means of the various strains ($F_{4,10}$ = 97.725, P < 0.0001). The Dunnett test was performed using the PoB strain as the reference. The results showed that there were significant differences between PoB vs T27 and T26 (Fig. 5). The decrease in the expression of the *lacc10* gene could be related to the results obtained in AGR. In this regard, Pezzella[22] reported that the laccase produced from *lacc10* seemed to play a significant role in the vegetative growth. This could be the reason why the transformants presented with problems of development during the vegetative growth of *P. ostreatus*, since the most strongly affected transformants showed a slower average growth rate compared to PoB, as shown by biomass production in solid culture by the T21, T26 and T27 transformants with respect to PoB. It has been suggested that in basidiomycetes, such as *A. bisporus,* the laccases that were silenced, such as the product of the *lacc10* gene, participate in mycelial development[11]. However, it is necessary to perform another type of analysis to answer these questions.

Biomass and Enzymatic Production in the Solid and Liquid Cultures

Figure 6 shows the biomass produced by the PoB and the transformants in the solid and liquid cultures. The ANOVA results indicate that there are significant differences between the strains with respect to the biomass obtained for both cultures ($F_{4,10}$= 79.6941, P < 0.0001 and $F_{4,10}$ = 26.8504, P < 0.0001 for solid and liquid cultures, respectively). Therefore, the Dunnett test was performed to analyze such differences between PoB and the transformants. For the solid culture, it was observed that the transformants T21, T26 and T27 showed significant differences compare to PoB. In other words, there was an effect in terms of the production of biomass in the transformants with medium and severe phenotypic alteration. In the liquid culture, no significant differences were found between the biomass of the T27 transformant with respect to PoB, while the T21, T26 and T7 transformants produced a biomass significantly higher than PoB.

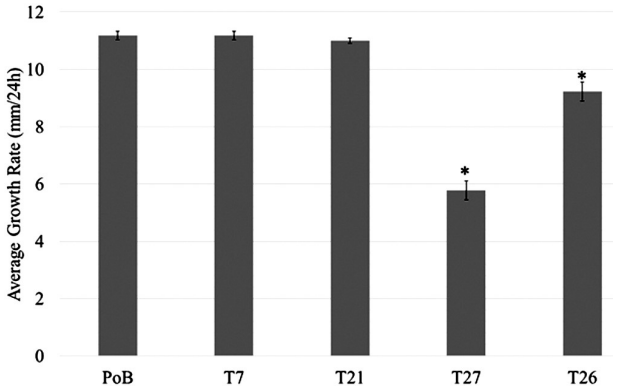


Fig. 5 —Average growth rate. Error bars are the standard error of three independent experiments and (*) indicates significant differences (Dunnett test $p < 0.05$).

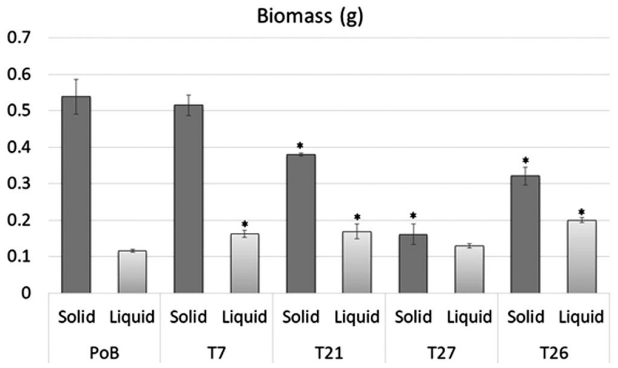


Fig. 6 — Biomass produced. Error bars are the standard error of three independent experiments and (*) indicates significant differences (Dunnett test $p < 0.05$).

Effect on Laccase Production

Figure 7 shows the effect on laccase production, as can be seen; the liquid culture produced more laccase activity than the solid culture. With respect to the analysis of variance of the solid culture, the results indicated that there were significant differences between the strains considering the specific activity obtained ($F_{4,10} = 70.946$, $P < 0.0001$). The Dunnett test was performed using PoB as the reference. The results show that there were significant differences between PoB vs T21, T26 and T27. In a similar way, the analysis of variance of the liquid culture indicated that there were significant differences between the strains with respect to the specific activity obtained ($F_{4,10} = 241.344$, $P < 0.0001$). Therefore, the post hoc Dunnett test was again performed, and it was determined that the T21, T26 and T27 transformants showed significant differences compare to PoB.

It was observed in the case of liquid culture the biomass produced by the transformants T21 and T26, was greater than the produced by PoB, but

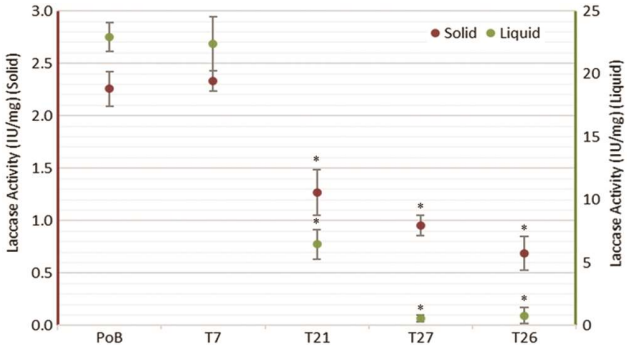


Fig. 7 — Specific laccase activity: a) solid and b) liquid cultures.

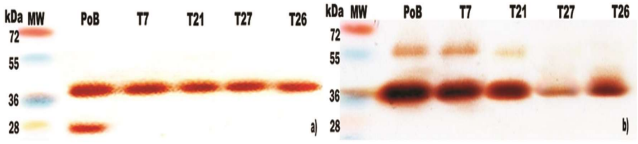


Fig. 8 — Number of isoenzymes obtained by zymography: a) solid and b) liquid cultures.

their enzymatic activity was lower. Based on a transcriptional analysis, it was reported, that there is not a proportional relationship between the amount of laccase activity and the production of biomass[21]. This suggests that the laccases silenced in these transformants do not have a primordial role in the production of biomass for this strain of *P. ostreatus* in liquid cultures. This finding can be the result of the presence of other enzymes that are compensating for the decrease in the production of laccase enzymes in the transformants, to facilitate the assimilation of the carbon source in the culture if the laccases are not performing that function. For example, this fungus also produces a versatile peroxidase (VP) encoded by *mnp4*, which is one of the nine members of the MnP gene family that forms part of the ligninolytic system of *P. ostreatus*[34].

On the other hand, there was a higher level of production of laccase activity in liquid cultures versus solid cultures. It has been reported that in solid cultures there is a down regulation of laccases genes' expression but the elements responsible for these responses are unknown. Figure 8 shows the number of isoenzymes obtained in solid (a) liquid cultures and (b) according to the molecular weight marker, an isoenzyme located at approximately 36–37 kDa was observed in both cultures and all of the strains (PoB and transformants). In the solid culture, an isoenzyme of approximately 25 kDa was produced, while in the liquid culture, a larger isoenzyme was produced measuring approximately 55 kDa. A decrease in the intensity of the constant isoenzyme was observed in

both culture systems. The second isoenzyme present in the solid culture from PoB disappeared in all of the transformants, while the larger isoenzyme observed in the liquid culture from the PoB was attenuated in the T7 and T21 transformants and disappeared in the T27 and T26 transformants.

These results can be correlated with the attenuation observed on the transcript *lacc10*, in the transformants with medium and severe phenotypic alteration. It has been suggested that the high transcriptional response of *lacc10* gene is very important to the induction of extracellular enzymatic activity[22]. Also, there could also be a relationship between the isoenzyme located at approximately 25 kDa and this gene; however, to confirm this, more data must be obtained about the produced isoenzyme, such a purification of that protein and obtaining its amino acid sequence. It would also be interesting to carry out the cultivation of the transformants in wheat straw to further observe the role of laccase enzymes in the morphology and development of the *Pleurotus ostreatus*.

Acknowledgements

This research was supported by the SEP-CONACyT basic science background for the "Physiological implication of gene inactivation of laccases in *Pleurotus ostreatus* PoB on morphogenesis and utilization of complex carbon sources" No. 167759. This work was also supported by a graduate fellowship from CONACyT (208531) received by Anahí Armas Tizapantzi.

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