SCREENING OF *LENTINULA EDODES* STRAINS BY LACCASE INDUCTION AND RESISTENCE TO *TRICHODERMA* SP.

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ABSTRACT

Production of laccase (E.C.1.10.3.2) and its induction by adding water soluble lignin derivatives obtained from Indulin AT, were studied in two succesive cultures of 39 strains of *Lentinula edodes*. Enzymatic activity was measured by oxidation of ABTS in the second culture and the inductor effect on mycelial growth was evaluated. The antagonism between a strain of *Trichoderma* sp. and 54 strains of *L. edodes* was tested *in vitro*. Resistance of *L. edodes* to *Trichoderma* invasion was shown by the formation of a brown line at the contact zone of the two fungi. The ability for laccase production of *L. edodes* strains is not corresponding to their ability for *Trichoderma* sp. rejection. This study shows diversity among *L. edodes* strains that could be applied to a program of selection and improvement.

Key words: induction, laccase, polyphenol oxidases, *Lentinula edodes*, *Trichoderma* sp., antagonism, edible mushroom.

RESUMEN

SELECCIÓN DE CEPAS DE *LENTINULA EDODES* POR LA INDUCCIÓN DE LA PRODUCCIÓN DE LACASA Y RESISTENCIA A *TRICHODERMA* SP. Rev. Mex. Mic. 14: 29-32 (1998). Se estudió la producción de lacasa (E.C. 1.10.3.2) y su inducción por la adición de derivados solubles de la lignina, obtenidos a partir de Indulina AT, en 2 cultivos sucesivos de 39 cepas de *Lentinula edodes*. La actividad enzimática de las cepas se evaluó a través de la oxidación de ABTS en el segundo cultivo. Se determinó el efecto del inductor en el crecimiento micelial de las cepas. Se estudió además la reacción antagónica entre una cepa de *Trichoderma* sp. y 54 cepas de *L. edodes*, por la formación de una línea obscura entre los micelios de los dos hongos. La capacidad de las cepas de *L. edodes* para producir lacasa en los medios de cultivo estudiados no corresponde con su aptitud para rechazar el ataque de *Trichoderma* sp. Sin embargo, este estudio muestra la existencia de una diversidad que puede ser aplicada a un programa de selección y mejoramiento de cepas.

Palabras clave: inducción, producción de lacasa, *Lentinula edodes*, *Trichoderma* sp., antagonismo, hongos comestibles.

Introducción

Lentinula edodes (Berk.) Pegler, the popular Japanese shiitake, is the second most important mushroom among the industrially cultivated species and its cultivation has recently started in Mexico. It has been cultivated for a long time in Asia, traditionally on oak logs, but there is a trend to use sterilized agricultural and forest by-products in order to reduce incubation time and increase yield (Royse *et al.*, 1985). Shiitake is a white rotting fungus which can be cultivated on different ligno-cellulosic substrates, depending on local availability (Mata & Gaitán-Hernández, 1992). Whatever the cultivation substrate used, competition with *Trichoderma* spp. remains one of the largest production problems. However, strains of *L. edodes* are able to reject the attack off *Trichoderma* under favorable temperature and nutritive conditions (Ishikawa *et al.*, 1980; Tokimoto, 1980, 1982). Production of a brownish

pigment in the contact zone between the hyphae when *L. edodes* rejects the attack of *Trichoderma* is commonly observed both in laboratory cultures and in commercial substrates. High levels of polyphenol-oxidase (PPO) activity in the browning area of interaction have been reported (Tokimoto, 1980, 1982). Otherwise PPO are implicated in lignin biodegradation (Bourbonnais & Paice, 1990). The most important PPO produced by shiitake is laccase (E.C. 1.10.3.2) (Leatham & Stahman, 1981).

The present study is part of a research on the mechanisms of shiitake adaptation to different cultivation substrates in order to define parameters for strain selection and to propose improvements in the substrates. The aim of this study was to use a large number of strains, in order to evaluate the potential of variability for laccase production, its induction by water soluble lignin derivatives and the brown line formation during the antagonism with *Trichoderma* sp.

Materials and methods

Strains and culture media. A *Trichoderma* strain was isolated from a contaminated sample of shiitake growing on wheat straw. It was identified as *T. harzianum* as defined by Rifai (1969), but in the absence of a confirmed identification, the strain was called *Trichoderma* sp. The strains of *L. edodes* used were from INRA (France) and Instituto de Ecologia (Mexico). They included cultivars, strains from international collections and hybrids produced in our laboratory. Strains were maintained on malt extract (2.0 %) agar (1.5 %) at 4°C. Mycelium on agar discs (∅ 5 mm) were used for a pre-cultivation at 28°C on malt agar and two pre-cultures were performed. Solid YMEA medium (2 % malt extract, 0.2 % yeast extract, 1.5 % agar) was used as a basic medium. Water soluble lignin derivatives (WSLD) were prepared by boiling 4 g of Indulin AT (Sigma) in 1 *l* as in Mata *et al.* (1997). After cooling and filtering on filter paper, the filtrate was diluted to adjust phenol concentration at 1 mM and the ingredients of the YMEA medium were added. After sterilization (20 min., 121°C), the medium was poured into 90 mm diameter Petri dishes.

Antagonism with *Trichoderma* sp. The ability of 54 strains of *L. edodes* to reject the attack of *Trichoderma* was determined *in vitro* on YMEA medium. *L. edodes* was cultivated for 5 days. Afterwards, inocula of *Trichoderma* sp. were placed on the Perti dishes at a distance of 4 cm from the inocula of *L. edodes*. The production of a brownish pigment at the contact zone between the mycelia and its apperance were observed 2 days after the inoculation of *Trichoderma* sp.

Enzyme and mycelial growth assays. For laccase production and mycelial growth, inoculum discs (∅ 5 mm) of 39 strains were removed from pre-cultures on malt-agar and placed in the center of the Petri dishes with YMEA, and supplemented or not supplemented with WSLD. Two cultures were performed; the first mycelial growth on YMEA or supplemented YMEA was named culture 1. Cultures 2 were obtained from inoculum discs of seven days old mycelium from cultures 1 on the same medium. Ten replicates were made with each strain and treatment. Mycelial growth rate was estimated seven days after inoculation by measuring two diameters of the colony, one taken perpendicularly from the other. Changes in mycelial growth rate from culture 1 to culture 2 were estimations of the adaptability of each strain (Mata *et al.*, 1997). Oxidation of ABTS [2,2'azino-bis (3 - ethylbenzthiazoline - 6 - sulfonic acid)] by one agar disc (∅ 5 mm) taken off in the growing area of mycelial colonies at seven days was determined on cultures 2 to estimate laccase activity (Mata *et al.*, 1997). One unit of laccase activity was 1 µM of ABTS oxidized /min/ mycelium disc. It was calculated by using $\varepsilon = 29300\ M^{-1}\ cm^{-1}$ (Niku-Paavola *et al.*, 1990). Two mycelial discs per culture and 10 cultures were tested by treatment the 39 strains studied. Data were then presented as the mean and standard deviation of 20 replicates. With laccase production data, a unifactorial analysis of variance was made and Tukey's multiple comparison method was used to determine the similarity between the means (P=95%) under each of the tested conditions.

Results and discussion

Among the 54 strains of *L. edodes* tested for their ability to reject *Trichoderma* attacks, only 3 strains were invaded by *Trichoderma* sp. The brown line at the contact zone between the colonies was dark for 25 strains and clear for 26 strains. Laccase activity in axenic cultures was measured for 39 of these strains. They were classified for their reaction to

Trichoderma sp. attacks into three groups (no line, clear and dark line). The means of laccase activities between these groups of strains were not significantly different (α=0.05) (Table 1).

Although supplementation of the YMEA medium with WSLD increased laccase activities (Table 1), this was not significant (α=0.05) for 4 of the 39 strains. The lowest activities were obtained with the same strain on both media, 0.03 ± 0.03 U and 0.17 ± 0.05 U on YMEA and YMEA + WSLD respectively. The maximum values were 8.85 ± 2.00 U and 12.90 ± 1.15 U on YMEA and YMEA + WSLD respectively.

Brown pigment at the contact zone with *Trichoderma*	Number of strains	Laccase activity (U) on YMEA	YMEA + WSLD
No	2	1.0 (0.53)*	1.2 (0.62)
Clear	18	1.5 (2.02)	4.7 (3.64)
Dark	17	0.9 (0.74)	4.0 (1.68)
undetermined	2	0.7 (0.01)	5.4 (0.81)

Table 1. Laccase activity of 39 strains of *L. edodes* classified for their ability to produce a brown line and to reject the attack of *Trichoderma* sp. * Values are means and (standard deviations).

Mycelial growth rates increased between cultures 1 and cultures 2 for both YMEA and YMEA + WSLD media (Table 2). However, the differences between cultures 1 and cultures 2 were not significant (α=0.05) for 7 strains on YMEA, for 4 strains on YMEA + WSLD, and for 3 strains on both YMEA and YMEA + WSLD. Supplementation of YMEA with WSLD decreased the overall mycelial growth rate on both cultures 1 and 2 (Table 2). However, WSLD had no significant effect (α=0.05) for 6 strains on culture 2 and their mycelial growth rate on YMEA was higher or not significantly different to the overall means. On the other hand, the ratios of laccase activity on YMEA + WSLD to laccase activity on YMEA ranged from 1.0 to 16.5 with a mean of 5.3 for the 39 strains tested.

Laccase is supposed to participate in lignin degradation and to detoxify phenolic compounds (Bourbonnais & Paice, 1990). Peaks of secretion of laccase activities into the wood log matrix or other substrates had been reported as being related to the early mycelial growth of *L. edodes* (Leatham, 1985; Mata & Savoie 1998; Savoie *et al.*, 1998). Improvement of strains by breeding is a way to obtain higher efficiency of substrate colonization and exploitation. An important variability of the ability for detoxification of phenolic compounds by laccase production was observed in the present study. Strains with high growth rates and a good adaptability to the presence of WSLD were identified. On the other hand, some strains appeared to be more dependent on an adaptation period for their ability to grow in the presence of WSLD with significant lower growth rates in cultures 1 than in 2.

Laccase production by *L. edodes* has also been associated with the formation of an antagonistic zone in dual cultures (Ohmasa *et al.*, 1991) or in interaction with competitive *Trichoderma* (Ishikawa *et al.*, 1980; Tokimoto, 1982). Despite differences in the levels of laccase activities measured on YMEA or YMEA with WSLD for the strains of *L. edodes* studied, no links with brown line formations and rejection of *Trichoderma* sp. were observed. Stimulation of laccase is a part of the reaction mechanism (Tokimoto, 1980; 1982), but the reaction, in this study, was not tied with the ability to produce laccase in axenic cultures. After the formation of the brown lines in the contact zones between the mycelia in the cultures, mechanisms other than laccase production could be implicated in the antagonic reaction. Savoie *et al.* (1998) proposed that fungistatic compounds produced by *Trichoderma* sp. stimulated the production of extracellular laccases and the formation of brown pigments in *L. edodes* hyphae by activating some general cellular mechanisms of stress resistance. The results suggest

Number of the culture	Culture media	
	YMEA	YMEA + WSLD
Culture 1	4.2 (0.72)	3.6 (0.62)
Culture 2	4.6 (0.71)	4.0 (0.82)

Table 2. Mycelial growth rate (cm 7 d^{-1}) of 39 strains of *L. edodes* on two successive cultures with or without water soluble lignin derivatives (WSLD). * Values are means and (standard deviations).

that mycelial growth rate and laccase production in presence of WSLD and the ability to reject *Trichoderma* attacks are two independent and important traits to be selected in a breeding program.

Acknowledgements

The authors are grateful to Delphine Dubreuil for her technical work. Dr. Gastón Guzmán and Msc. Rigoberto Gaitán-Hernández revised this work. G. Mata wishes to thank CONACYT (Mexico) for funding his training period in France.

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Recibido: 17 de junio, 1997. Aceptado: 14 de septiembre, 1998.
Solicitud de sobretiros: Gerardo Mata.