TAMARA LEITE DOS SANTOS

Physiological, biochemical and molecular characterization of the brown film of *Lentinula edodes* (Berk.) Pegler and its interaction with *Trichoderma* sp.

LAVRAS – MG

2019

TAMARA LEITE DOS SANTOS

Physiological, biochemical and molecular characterization of the brown film of *Lentinula edodes* (Berk.) Pegler and its interaction with *Trichoderma* sp.

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

Prof. Dr. Eduardo Alves
Orientador

Prof. Dr. Eustáquio Souza Dias
Coorientador

LAVRAS – MG
2019

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Santos, Tamara Leite dos.

Physiological, biochemical and molecular characterization of the brown film of *Lentinula edodes* (Berk.) Pegler and its interaction with *Trichoderma* sp. / Tamara Leite dos Santos. - 2019.

73 p. : il.

Orientador(a): Eduardo Alves.

Coorientador(a): Eustáquio Souza Dias.

Tese (doutorado) - Universidade Federal de Lavras, 2019.

Bibliografia.

1. Atividade enzimática. 2. Microscopia eletrônica. 3. Expressão gênica. I. Alves, Eduardo. II. Dias, Eustáquio Souza. III. Título.

TAMARA LEITE DOS SANTOS

Physiological, biochemical and molecular characterization of the brown film of *Lentinula edodes* (Berk.) Pegler and its interaction with *Trichoderma* sp.

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

APROVADA em 25 de Fevereiro de 2019.

Drª. Maiara Andrade de Carvalho Sousa	MAPA
Drª. Lívia Martinez Abreu Soares Costa	UFLA
Dr. Silvino Intra Moreira	UFLA
Drª. Suzana Reis Evangelista	UFLA

Prof. Dr. Eduardo Alves
Orientador

Prof. Dr. Eustáquio Souza Dias
Coorientador

LAVRAS – MG
2019

AGRADECIMENTOS

À Universidade Federal de Lavras (UFLA), em especial ao Programa de Pós-graduação em Microbiologia Agrícola, pela oportunidade de aperfeiçoamento concedida.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

À CAPES pela concessão da bolsa de estudos no Brasil e no exterior.

À Fapemig, pelo apoio financeiro na manutenção de equipamentos do Laboratório de Microscopia Eletrônica e Análise Ultraestrutural (LME) da UFLA.

Ao professor Dr. Eduardo Alves pelo suporte e confiança durante os 6 anos de trabalho.

Ao meu coorientador Dr. Eustáquio Souza Dias pela disponibilidade e colaboração nessa parceria.

À Universidad Publica de Navarra (UPNA), Pamplona, Espanha, pela oportunidade de realizar o "doutorado sanduíche". Em especial ao meu coorientador Dr. Antonio Gerardo Pisabarro e à Drª Lucía Ramirez Nasto, por todo o apoio e confiança em mim depositada.

À Drª Maria Gumersinda Perez Garrido e ao Dr. Raúl Castanera Andrés pela amizade e pelo valioso auxílio nos trabalhos.

A toda a minha família, por permanecer sempre ao meu lado durante esta jornada, em especial minha mãe, Irani; meu pai, João e meu irmão Renan.

Ao meu noivo Jorge, por todo o companheirismo, paciência, ajuda e carinho!!

Às técnicas Elisa e Aline, pela atenção, dedicação e amizade durante esta caminhada.

Ao técnico Paulinho, por toda ajuda.

À secretária Rose, pela paciência e prestatividade.

A todos os meus amigos do Laboratório de Cogumelos Comestíveis e do LME por toda ajuda, troca de experiências e pela convivência harmoniosa.

Aos amigos: Raúl, Alessandra, Manuel, Elvira, Iñaki e Jose Luiz, pelo acolhimento durante minha estadia em Pamplona. Em especial, minha amiga Shirley, que ao final se tornou uma irmã!!!

A todos que de alguma forma caminharam comigo nesta jornada, minha eterna gratidão!!!

RESUMO

O ciclo de cultivo do shiitake apresenta várias etapas, sendo a formação da capa marrom a mais importante, visto que, é um passo estratégico na produção de cogumelos. Muitos estudos já demonstraram a relação da atividade de algumas enzimas na formação da capa marrom, principalmente as ligninases. Outro aliado no entendimento deste processo é o estudo da expressão gênica de linhagens de *L. edodes* que se comportam de maneira diferente durante a formação da capa marrom. Durante a produção de cogumelos, um fator muito importante é a contaminação, sendo o fungo do gênero *Trichoderma* o mais estudado. O controle deste patógeno é realizado através da adoção de técnicas assépticas, entretanto, sabe-se que a capa marrom também pode auxiliar no controle, atuando como barreira física. Neste sentido, a microscopia eletrônica de varredura (MEV) é uma técnica poderosa no estudo da interação hospedeiro-patógeno. Dada a relevância da formação da capa marrom no shiitake, neste estudo foi investigada a dinâmica desse processo em nível fisiológico, bioquímico e molecular. Nossos resultados mostraram que todas as enzimas apresentaram atividade em diferentes condições de cultivo, sendo que a lignina peroxidase apresentou a maior atividade quando comparada a outras enzimas para as duas linhagens. Além disso, a alta atividade de tirosinase na linhagem UFLA-LE6 já no começo do cultivo, pode explicar o fato desta linhagem formar a capa marrom mais rapidamente que a UFLA-LE5 e apresentar altos níveis de produtividade. Entretanto, observamos que esta mesma linhagem é mais suscetível a mudanças ambientais, como temperatura e pH; diferente da linhagem UFLA-LE5, a qual apresentou uma maior amplitude de crescimento em ambientes considerados atípicos para um bom desenvolvimento. Na questão do antagonismo com *Trichoderma* sp., a cepa UFLA-LE6 produziu uma linha marrom na zona de interação com o patógeno, o que já foi relatado como uma estratégia de resistência e está provavelmente relacionado com a síntese de melanina. Com a MEV, detectamos um aumento da infecção ao longo do tempo, a penetração direta do patógeno no cogumelo, além de indícios de micoparasitismo. Na análise transcriptômica vimos que a linhagem UFLA-LE5 tem mais genes super-expressos comparado com a linhagem UFLA-LE6. Este fato pode parecer contraditório, no entanto, pode sugerir uma falha desta linhagem em controlar o nível da expressão genética. Alguns dos genes estudados com a RT-qPCR exibiram uma diminuição da expressão na amostra externa da cepa UFLA-LE6 aos 90 dias. Esse perfil pode ser explicado pela velocidade e estabilidade com que a capa marrom é formada nessa linhagem, dessa maneira, após esse processo tais genes podem não ser mais requeridos e, consequentemente não expressos.

Palavras-chave: Cogumelo. Atividade enzimática. Microscopia eletrônica. Expressão gênica.

ABSTRACT

The cultivation cycle of shiitake has several stages and the formation of brown film is the most important, since it is a strategic step in the production of mushrooms. Many studies have already shown the relation of the activity of some enzymes in the brown film formation, mainly the ligninases. Another ally in the understanding of this process is the study of gene expression of *L. edodes* strains that behave differently in the brown film formation. During the production of mushrooms, a very important factor is the contamination, being the fungus of the genus *Trichoderma* the most studied. The control of this pathogen is accomplished through the adoption of aseptic techniques, however, it is known that the brown cover can also aid in the control, acting as a physical barrier. In this sense, scanning electron microscopy (SEM) is a powerful technique in the study of host-pathogen interaction. Given the relevance of the brown film in shiitake, this study investigated the dynamics of this process at the physiological, biochemical and molecular levels. Our results showed that all the enzymes showed activity in different culture conditions, and the lignin peroxidase presents the highest activity when compared to other enzymes, for both strains. In addition, the high activity of tyrosinase in the UFLA-LE6 strain at the beginning of the cultivation may explain the fact that this strain forms the brown film faster than UFLA-LE5 strain and presents high levels of productivity. However, we observed that this same strain is more susceptible to environmental changes, such as temperature and pH; different from the UFLA-LE5 strain, which presented a greater range of growth in environments considered atypical for a good development. On the issue of antagonism with *Trichoderma* sp., the UFLA-LE6 strain produced a brown line in the zone of interaction with the pathogen, which has already been reported as a resistance strategy and is probably related to melanin synthesis. With SEM we detected an increase in infection over time, direct penetration of the pathogen in the mushroom and evidence of mycoparasitism. In the transcriptomic analysis, we have seen that the UFLA-LE5 strain has more upregulated genes compared to the UFLA-LE6 strain. This fact may seem contradictory; however, it may suggest a failure of this strain to control the level of genetic expression. Some of the genes studied with RT-qPCR exhibited a decrease in expression on the outside sample of UFLA-LE6 strain at 90 days. This profile can be explained by the speed and stability with which the brown film is formed in this strain, so, after that process such genes may no longer be required and therefore not expressed.

Keywords: Mushroom. Enzymatic activity. Electron microscopy. Gene expression.

SUMÁRIO

1 INTRODUÇÃO

O cogumelo *Lentinula edodes*, também conhecido como shiitake é o cogumelo mais consumido no Brasil e no mundo. Seu cultivo pode ser realizado de duas maneiras, através do método tradicional em toras ou em cultivo axênico. Essa técnica tem como base a produção de cogumelos em blocos sintéticos compostos majoritariamente por serragem e suplementados com farelos. A produção do shiitake gira em torno de 3 meses e apresenta várias etapas, sendo a formação da capa marrom a mais importante.

Muitos estudos já demonstraram que uma capa micelial bem estabelecida pode ocasionar uma alta produtividade de cogumelos. Além disso, sabe-se que há uma relação entre algumas enzimas, como lacase, lignina peroxidase, manganês peroxidase e tirosinase com a formação da capa marrom. Portanto, é de extrema importância a investigação destas atividades enzimáticas durante esse processo.

O estudo da expressão gênica de linhagens de *L. edodes* que se comportam de maneira diferente durante a formação da capa marrom, pode contribuir para um maior conhecimento dos genes envolvidos no processo. Diante disso, a comparação de perfis de expressão através da análise transcriptômica e a validação dessas diferenças quantitativas usando a RT-qPCR possibilitam a dedução dos níveis de expressão durante o ciclo de cultivo.

Outro fato importante que deve ser levado em consideração durante a produção de cogumelos, é a contaminação. Dentre os vários patógenos conhecidos, as espécies de *Trichoderma* são recorrentes e de difícil eliminação, visto que estão presentes naturalmente no solo. O controle desse patógeno é feito através de técnicas assépticas durante todo o cultivo, além disso, em alguns países é permitido o uso de fungicidas. Neste sentido, uma capa marrom bem formada e estabelecida age como uma barreira física, atuando no controle de patógenos. A microscopia eletrônica é uma ferramenta poderosa no estudo da interação hospedeiro-patógeno, proporcionando informações a respeito da adesão e colonização desses fungos.

Dada a relevância da formação da capa micelial marrom para a formação de corpos de frutificação em shiitake, é importante investigar a dinâmica desse processo em nível fisiológico, bioquímico e molecular. Assim, o presente trabalho foi realizado com os seguintes objetivos: i) verificar a produção de ligninases e tirosinase, ao longo do ciclo de cultivo de duas linhagens de *L. edodes*; ii) avaliar o crescimento micelial de cepas de *L. edodes* e *Trichoderma* sp. sob diferentes condições de cultivo; iii) analisar o antagonismo *in vitro* de *L. edodes* e *Trichoderma* através da microscopa eletrônica de varredura; iv) determinar o nível de expressão gênica de transcritos super-expressados em duas linhagens de *L. edodes* ao longo do tempo; v) verificar as diferenças de expressão destes genes em diferentes estágios de crescimento.

2 REFERENCIAL TEÓRICO

2.1 *Lentinula edodes*

O shiitake é um fungo aeróbio decompositor primário de madeira, cientificamente denominado *Lentinula edodes* (Berk.) Pegler, e pertencente à classe dos basidiomicetos (CHANG; MILES, 2004). Apesar de já ter sido referido por uma variedade de nomes científicos, Berkley foi quem primeiro descreveu a espécie como sendo pertencente ao gênero *Lentinus* em 1941 e posteriormente Pegler, em 1975, inseriu a espécie no gênero *Lentinula* (MINHONI et al., 2007).

Quanto às características morfológicas, o shiitake possui o píleo em forma convexa, circular ou reniforme, com 5 a 15 cm de diâmetro. A coloração da epiderme superior varia de ocre-claro a marrom escuro e o himênio de cor creme. O estipe é cilíndrico e varia de 3 a 7 cm de comprimento. As lamelas são aderidas ao estipe, e não possuem volva e véu (CHANG; MILES, 2004).

O shiitake representa hoje o cogumelo comestível mais consumido no mundo, seguido dos cogumelos pertencentes aos gêneros *Pleurotus* e *Auricularia* (ROYSE; BAARS; TAN, 2017). Além de apresentar um apreciável sabor, destaca-se também por suas propriedades terapêuticas e nutricionais, tornando-se conhecido como “elixir da vida” (MENOLLI JUNIOR; PACCOLA-MEIRELLES, 2010). Seu cultivo originou-se na China e hoje está distribuído naturalmente no Japão, Coréia, Filipinas, Papua Nova Guiné e Norte da Tailândia. No Brasil seu cultivo foi estabelecido principalmente nas Regiões Sul e Sudeste, devido às condições climáticas favoráveis (ISHIKAWA et al., 2001). Mundialmente, sua produção representa 25% do total produzido; sendo que nos últimos anos, foi a produção de cogumelos que mais cresceu (JIANG; LUO; YING, 2015).

2.2 Cultivo de shiitake

O shiitake é um fungo decompositor de madeira capaz de utilizar lignina, celulose e hemicelulose como fontes de carbono. Devido tais habilidades, seu cultivo pode ser feito em troncos de árvores como o eucalipto, carvalho, mangueira e abacateiro (ZHANXI; ZHANHUA, 2001). Alternativamente, foi desenvolvido também o sistema de cultivo axênico, segundo o qual o fungo é cultivado em substratos à base de serragem enriquecidos com farelos (farelo de trigo ou farelo de arroz) e esterilizados em autoclave (URBEN, 2004). Este sistema oferece maior rentabilidade quando comparado ao cultivo tradicional em toras, uma vez que a colheita acontece mais rapidamente e a eficiência biológica da produção é elevada (KALBERER, 2000). No entanto, o cultivo axênico é mais oneroso, pois envolve formulação de substrato

adequado, controle preciso das condições de crescimento e frutificação, e além disso, depende do genótipo da cepa empregada (CHEN; ARROLD; STAMETS, 2000).

As condições ambientais são extremamente importantes para o cultivo do shiitake. Dentre as variáveis ambientais, a temperatura é um dos fatores mais importantes, influenciando tanto o tempo de colonização do substrato como a produção do cogumelo. Para a frutificação, muitos cogumelos exigem temperaturas mais baixas em relação à temperatura ideal para o crescimento micelial. Para o shiitake, a indução da frutificação ocorre quando a temperatura do ambiente se situa na faixa de 15 a 20°C e com a umidade relativa do ar acima de 80% (CHANG; MILES, 2004; CHEN, 2005). Além disso, o shiitake apresenta baixa tolerância às temperaturas elevadas, típicas das estações mais quentes no Brasil (MENOLLI JUNIOR; PACCOLA-MEIRELLES, 2010).

O ciclo de cultivo pode ser dividido em quatro etapas, segundo Tang et al. (2013):

1) Crescimento micelial vegetativo;

2) Formação da capa micelial marrom;

3) Formação dos primórdios;

4) Desenvolvimento dos corpos de frutificação.

O crescimento micelial refere-se ao crescimento imediatamente após a inoculação, até a completa colonização do substrato pelo fungo. Nesta fase, o micélio absorve nutrientes, os quais serão utilizados na sua estrutura e na conversão da energia necessária para seu metabolismo e crescimento. A próxima etapa caracteriza-se pela estabilização e maturação do micélio, que vai até o endurecimento e o escurecimento da capa micelial, que se torna amarronzada. Posteriormente, a indução dos primórdios pode ser feita alterando-se a temperatura de incubação (± 24°C) por temperaturas mais baixas, entre 15 a 18°C, com o objetivo de estimular e acelerar a formação dos mesmos. E por fim, ocorre o desenvolvimento dos basidiomas a partir dos primórdios (CHANG; MILES, 2004).

2.3 Formação da capa micelial marrom

Esta etapa do ciclo de cultivo tem se mostrado um tema atraente e interessante, a partir do aspecto da pesquisa e produção, uma vez que a formação dessa capa pode ser considerada um passo de morfogênese peculiar do shiitake. Esta etapa precede a formação dos primórdios e, posteriormente, dos corpos de frutificação (TSIVILEVA et al., 2005; CHUM et al., 2008).

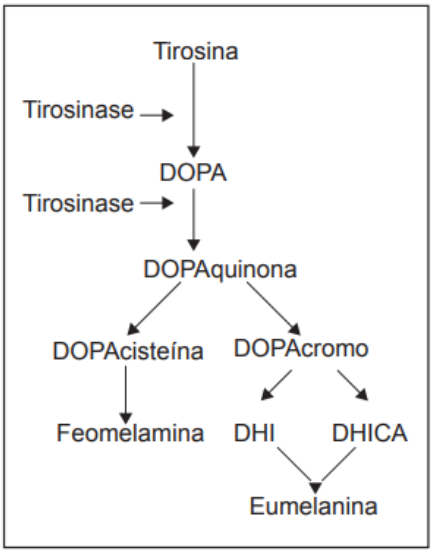
Sabe-se que a formação da capa micelial é muito importante, pois ela age como uma barreira à perda de umidade, sendo também uma defesa contra agentes tóxicos e patogênicos, além de proteger

contra a iluminação excessiva (ROYSE, 2009). No entanto, os mecanismos moleculares e bioquímicos da formação da capa micelial marrom ainda não foram totalmente elucidados (TANG et al., 2013).

Estudos apontam para a ligação entre a produção de um complexo de enzimas, em alguns fungos, incluindo *Lentinula edodes*, e a síntese de melaninas na formação de estruturas, tais como a capa micelial. É uma estrutura densa rica em pigmentos, que parece realizar uma função de proteção, no início da formação do corpo de frutificação, a qual é, muitas vezes, acompanhada pela atividade de fenol oxidases. Este processo, por sua vez, está intimamente relacionado com a formação de pigmentos extracelulares e prossegue em paralelo com a polimerização oxidativa dos componentes da parede celular e o reforço da adesão intercelular (LEATHAM; STAHMANN, 1981).

As melaninas são macromoléculas formadas por polimerização oxidativa de compostos fenólicos ou indólicos (Figura 1). Muitas vezes, o que resulta são pigmentos marrons ou pretos, mas muitas outras cores também foram observadas (LANGFELDER et al., 2003).

Figura 1 - Biossíntese da melanina.



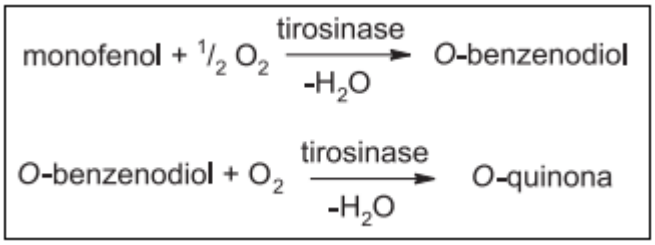
Fonte: Rocha e Moreira (2007).

Os dois tipos de melaninas mais importantes são: DHN-melanina (nome de um dos intermediários da via, 1,8-di-hidroxinaftaleno) e DOPA-melanina (nome de um dos precursores, L-3,4-dihidroxifenilalanina) (HAMILTON; GOMEZ, 2002). Tais compostos fenólicos são oxidados enzimaticamente a quinonas, que polimerizam por meios não enzimáticos para formar os pigmentos de melanina. A oxidação destes compostos fenólicos é, geralmente, catalisada por tirosinase (NAGAI et al., 2003).

2.4 Tirosinases

Tirosinases pertencem a um grande grupo de proteínas, cuja função é catalisar a hidroxilação de monofenóis a O-diidróxi fenóis e da subsequente oxidação a O-quinonas. Sendo o oxigênio o receptor de elétrons para ambas as reações (Figura 2). Posteriormente, as quinonas sofrem reações não enzimáticas, produzindo pigmentos castanho-escuros (STURM; TEASDALE; BOX, 2001).

Figura 2 - Reações catalisadas pela tirosinase.



Fonte: Marques e Yamanaka (2008).

As tirosinases são encontradas em uma vasta gama de organismos, incluindo microrganismos procariotos e eucariotos, plantas, invertebrados e mamíferos, e estão envolvidas em uma variedade de funções biológicas, como pigmentação da pele em mamíferos, escurecimento em plantas e cogumelos e sistemas de defesa em artrópodes (CLAUS; DECKER, 2006; WICHERS et al., 2003; WANG; HEBERT, 2006).

Vários estudos sobre esta enzima encontrada em fungos, tais como *Pleurotus sajor-caju* (MODA et al., 2005), *Lentinula edodes* (KANDA et al., 1996) e *Aspergillus oryzae* (NAKAMURA et al., 2000), já foram realizados. No entanto, durante as últimas décadas, os estudos foram essencialmente voltados para tirosinases do basidiomiceto *Agaricus bisporus*. Sendo, a maioria deles motivada pelo escurecimento enzimático do cogumelo durante o desenvolvimento e o armazenamento pós-colheita, o que, particularmente, diminui o valor comercial do produto (BRENNAN; LE PORT; GORMLEY, 2000; WICHERS et al., 2003; SAPERS et al., 2001).

2.5 Ligninases

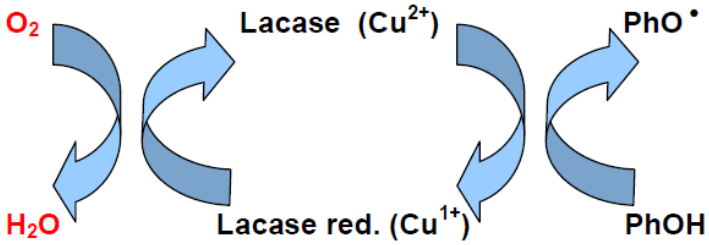
Lignocelulose é um complexo macromolecular, constituído de lignina, celulose e hemicelulose (HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012). Existem três tipos de fungos que efetivamente degradam um ou mais componentes da madeira: fungos de podridão-mole, fungos de podridão-marrom e fungos de podridão-branca. Os fungos de podridão-mole, assim como os de podridão-marrom, podem degradar a celulose, mas degradam a lignina apenas parcialmente (CANNON; KIRK,

2007). Já os fungos de podridão-branca, como o *L. edodes* (Berk) Pegler, são dotados de um sistema lignilítico constituído de ligninases, incluindo lacase (Lac), lignina peroxidase (LiP), manganês peroxidase (MnP), entre outras (ABRAHÃO et al., 2008). Estas enzimas são responsáveis pela geração de radicais livres altamente reativos e não específicos que contribuem para a degradação da lignina (MARTÍNEZ et al., 2005).

2.5.1 Lacase

Lacases pertencem à família das multicobre oxidorredutases que catalisam a oxidação de uma variedade de substratos aromáticos por meio da redução de oxigênio à água (PAOLA, 2010). O ciclo catalítico da lacase envolve quatro íons Cu^{2+}, normalmente ligados a uma única proteína ou a 2 cadeias protéicas acopladas, quatro substratos fenólicos, quatro prótons e uma molécula de oxigênio. Os quatro íons Cu^{2+} são reduzidos a quatro íons Cu^{1+} pela oxidação de quatro substratos fenólicos a radicais fenóxil. A lacase então reduzida transfere quatro prótons ao oxigênio molecular, reduzindo-o à água e, assim, a enzima retorna ao seu estado nativo (Figura 3).

Figura 3 - Ciclo catalítico da Lacase, PhOH = substrato fenólico.



Fonte: Adaptado de Aguiar e Ferraz (2011).

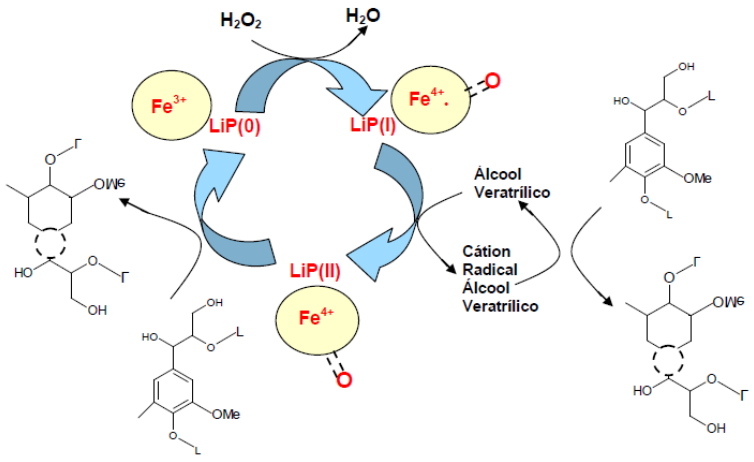
Essas enzimas são amplamente distribuídas e já foram encontradas e estudadas em fungos de podridão-branca, como *Lentinus tigrinus* (FERRARONI et al., 2007), *Pleurotus ostreatus* D1 (POZDNIAKOVA et al., 2006) e *Trametes pubescens* (SHLEEV et al., 2007). Estudos têm apontado para o envolvimento de lacase na formação do filme micelial marrom (TANG et al., 2013). A formação da capa micelial é acelerada pelo oxigênio e o envolvimento de lacase pode ser considerado pela formação de gotículas de água em consequência da oxidação de compostos fenólicos acoplada à redução de oxigênio à água (RODGERS et al., 2010). Estudos anteriores também ligaram lacases com o desenvolvimento do corpo de frutificação (CHEN; GE; BUSWELL, 2004; OHGA et al., 2000) e a produção de pigmentos em cogumelos comestíveis (LEATHAM; STAHMANN, 1981).

2.5.2 Lignina peroxidase

Lignina peroxidase é uma heme proteína classificada na classe II da superfamília de peroxidases de plantas, fungos e bactérias (RUIZ-DUEÑAS; MARTÍNEZ, 2010).

A enzima no seu estado reduzido (LiP0) é oxidada pelo peróxido de hidrogênio ao composto I (LiPI) deficiente em dois elétrons. O composto I (LiPI) é então reduzido ao composto II (LiPII) pela abstração de um elétron do substrato aromático não fenólico (WONG, 2009). A enzima volta ao seu estado completamente reduzido (LiP0) pela doação de mais um elétron do substrato aromático não fenólico (Figura 4).

Figura 4 - Ciclo catalítico da Lignina Peroxidase.



Fonte: Adaptado de Aguiar e Ferraz (2011).

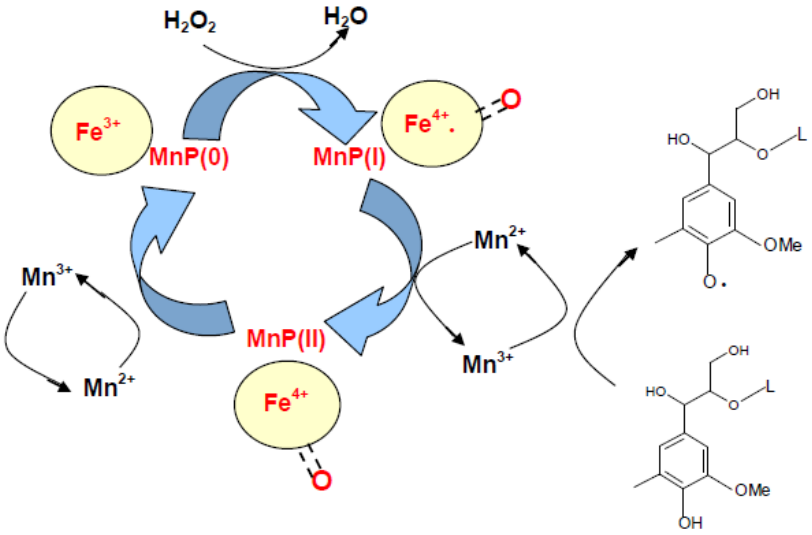
O álcool veratrílico é um metabólito secundário produzido por alguns fungos de decomposição branca e desempenha um papel importante no ciclo catalítico da LiP, atuando como redutor da enzima e aparentemente a protege da inativação por excesso de H_2O_2 (GOODELL, 2003). A oxidação da estrutura da lignina catalisada pela LiP ocorre por intermédio da oxidação do álcool veratrílico, resultando em uma ampla variedade de fragmentos de decomposição da lignina (KIRK; CULLEN, 1998).

Desde a sua descoberta em *Phanerochaete chrysosporium*, mais LiPs foram encontradas em diferentes cepas deste (RENGANATHAN; MIKI; GOLD, 1985; TIEN; KIRK, 1984) e de outros fungos de podridão-branca, como *Trametes versicolor* (JOHANSSON; WELINDER; NYMAN, 1993). Além disso, genes de LiP foram detectados em diferentes espécies de fungos, incluindo *Panus* sp., *P. coccineus*, *P. sanguineus* e *Perenniporia medula* (STEPHEN et al., 2005). *Lentinula edodes* apresentou atividade de LiP em meio contendo extrato de malte como fonte de carbono (ESPOSITO; CANHOS; DURDN, 1991).

2.5.3 Manganês peroxidase

Manganês peroxidades são glicoproteínas heme que têm a capacidade de catalisar a oxidação de Mn^{+2} para Mn^{+3} (TAKANO; NAKAMURA; YAMAGUCHI, 2010). MnPs também possuem um ciclo catalítico semelhante as LiPs, tal como mostra a figura 5, mas tendo Mn(II) como doador de elétron (WARIISHI et al., 1988). A MnP no seu estado reduzido (MnP0) é oxidada pelo peróxido de hidrogênio a um composto I (MnPI), o qual é um complexo oxo-porfirina-Fe^{4+} radical e pode oxidar substrato fenólico ou MnP(II) monoquelado por ácidos orgânicos tais como oxálico, cítrico ou fumárico. Esta oxidação reduz a enzima a um composto II (MnPll), agora formando um complexo oxo-porfirina-Fe^{4+} (HOFRICHTER, 2002). Por fim, o composto II (MnPII) é totalmente reduzido ao composto (MnP0) somente pela oxidação de MnP(II) monoquelado por ácido orgânico.

Figura 5 - Ciclo catalítico de Manganês Peroxidase.



Fonte: Adaptado de Aguiar e Ferraz (2011).

O íon Mn^{3+} quelado por ácidos orgânicos como oxálico, málico, malônico e lático é capaz de oxidar vários substratos através da abstração de um elétron. Fenóis são levados a radicais fenoxil, aminas aromáticas são transformadas em radicais amino, certos aromáticos não-fenólicos são levados a radicais cátions arílicos, ácidos carboxílicos a superóxidos, tióis a radicais altamente reativos (HOFRICHTER, 2002).

Desde a descoberta de manganês peroxidase em *Phanerochaete chrysosporium* (GLENN; GOLD, 1985), mais MnP foram encontradas em basidiomicetos, tais como *Panus tigrinus* (LISOV; LEOTIEVSKY; GOLOVLEVA, 2003), *Lenzites betulinus* (HOSHINO et al., 2002), *Phanerochaete flavido-alba* (DE LA RUBIA et al., 2002), *Agaricus bisporus* (LANKINEN et al., 2001) e *Nematoloma*

frowardii b19 (HILDEN et al., 2008). Além disso, alguns estudos tem descrito *Lentinula edodes* como um bom produtor de manganês peroxidase (BOER et al., 2006; SAKAMOTO et al., 2009).

2.6 Contaminantes na produção do shiitake

A produção de shiitake, tanto no sistema de cultivo em toras como em substratos sintéticos, tem sido atingida por uma ampla gama de pragas e doenças, principalmente as espécies do gênero *Trichoderma*, as quais têm causado sérios problemas à produção (LEE et al., 2008). Estes fungos são comumente encontrados no solo, uma vez que são muito utilizados como agentes de controle biológico de fungos fitopatogênicos (CHEN et al., 1999; OSPINA-GIRALDO et al., 1999) e também em madeiras onde causam manchamento (LAZAROTTO et al., 2016). Além disso, produzem uma grande variedade de metabólitos secundários que apresentam atividades inibitórias, micoparasitismo direto, bem como lise e degradação da parede celular (HOWELL, 2003).

Terashima, Igusa e Ohga (2002), estudando o efeito dos contaminantes *Penicillium brevicompactum* e *Trichoderma harzianum*, durante a colonização do substrato de cultivo e a formação dos corpos de frutificação, demonstrou que algumas linhagens de *L. edodes* interromperam seu crescimento quando entraram em contato com os contaminantes, enquanto as linhagens que continuaram a crescer colonizaram o substrato, mas produziram corpos de frutificação irregulares. Da mesma maneira, constatou-se que a contaminação pelas espécies *T. harzianum* e *Trichoderma polysporum*, reduziu drasticamente o rendimento do cultivo de shiitake (SEABY, 1998; TOKIMOTO, 1985; ULHOA; PERBERDY, 1992).

O controle desse patógeno depende de práticas assépticas rigorosas, juntamente com a aplicação de fungicidas, como benomyl, nos países onde esta prática é permitida; uma vez que os esporos são facilmente transportados por trabalhadores, insetos e equipamentos contaminados (CHEN, 1998). Contudo, relatos sobre resistência a fungicidas em *Trichoderma* sp. já foram descritas, o que estimula o desenvolvimento de novas estratégias de manejo do patógeno (GROGAN et al., 1996, 1997). Por outro lado, um entendimento mais aprofundado sobre adesão, penetração e colonização nos permite adotar novas formas de combate ao patógeno. Neste contexto, Alves et al. (2013) ressaltam que, para a compreensão da relação entre patógeno-hospedeiro, a microscopia eletrônica, de modo geral, tem proporcionado inestimáveis contribuições.

2.7 Análise de expressão gênica

O estudo da expressão gênica de linhagens de *L. edodes* que se comportam de forma diferente durante a colonização do substrato de cultivo do cogumelo e, principalmente, durante a formação da capa marrom, a qual precede o início da frutificação do cogumelo, pode contribuir para o melhor conhecimento dos genes envolvidos no processo.

2.7.1 Análise transcriptômica

A capacidade de medir simultaneamente a expressão de milhares de genes é um sistema analítico poderoso, e a disponibilidade de novas tecnologias para esse fim tem fornecido novas estratégias de estudo da resposta gênica (MITRA et al., 2003). Nos últimos anos, o perfil do transcriptoma tem sido usado para obter informações de diferentes genes durante o ciclo de desenvolvimento de *L. edodes*. Desse modo, várias técnicas foram adotadas para este propósito, incluindo a reação em cadeia da polimerase em tempo real (RT-qPCR) (LEE et al. 2001), análise de diferença representacional (RDA) (MIYAZAKI; NAKAMURA; BABASAKI, 2005), análise serial da expressão gênica (SAGE) (SUIZU et al., 2008; CHUM et al., 2008), hibridação subtrativa por supressão (SAKAMOTO; NAKADE; SATO, 2009), pirosequenciamento e Long-SAGE (CHUM et al., 2011).

As tecnologias utilizadas para analisar o perfil transcricional permitem a análise de populações de RNAm a partir de células ou tecidos selecionados, produzindo medidas de expressão gênica em larga escala. No entanto, os dados fornecidos podem ser utilizados e interpretados de diversas maneiras (MEYERS et al., 2004). Ou seja, nenhuma das técnicas existentes suporta todas as necessidades experimentais, havendo vantagens e desvantagens para cada uma delas, porém, tais diferenças as fazem complementares (CLOSE, 1996).

Recentemente, com o rápido desenvolvimento de tecnologias de sequenciamento profundo de alto rendimento como o RNA-seq da Illumina, o perfil de expressão genômica tem sido aplicado a vários fungos (NAGALAKSHMI et al., 2008; WANG et al., 2010; YU et al., 2011). O RNA-seq é uma abordagem rápida, econômica e de alto rendimento para gerar grandes números de leituras curtas de alta qualidade para obter dados transcriptômicos abrangentes, e é particularmente atraente para a análise de transcriptoma “De Novo” de organismos sem genoma de referência (WONG; CANNON; WICKNESWARI, 2011; LI et al., 2012).

2.7.2 PCR quantitativo em tempo real (RT-qPCR)

Devido à sua alta sensibilidade, reprodutibilidade e especificidade, a RT-qPCR representa uma das técnicas mais amplamente utilizadas para quantificar níveis de expressão gênica (HAYWARD-LESTER et al., 1995; GINZINGER, 2002). Os sinais de fluorescência são gerados por fluoróforos, como o "SYBR Green", que são específicos para a fita dupla de DNA (cDNA) ou por "primers" marcados por fluorescência em regiões específicas (KLEIN et al., 2002). O sinal é proporcional à quantidade de produto de PCR e o equipamento detecta o acúmulo do produto amplificado durante cada ciclo da reação (BUSTIN, 2000).

A obtenção de resultados precisos e confiáveis depende de vários fatores, incluindo a qualidade e a quantidade do RNA inicial, especificidade dos primers, eficiência da transcrição reversa, eficiência da amplificação, condições da PCR e normalização dos dados; sendo, este último, o mais importante (CZECHOWSKI et al., 2005). Genes "House-keeping" são geralmente expressos em níveis estáveis sob várias condições, e, consequentemente, considerados genes de referência eficazes. No entanto, alguns estudos já relataram que a estabilidade desses genes pode variar sob uma gama de condições experimentais (NICOT et al., 2005; EXPÓSITO-RODRÍGUEZ et al., 2008). Estudos de seleção e avaliação de genes de referência já foram realizados em várias espécies (WAN et al., 2010; XU et al., 2011; YOU et al., 2016). Entretanto, estudos com *L. edodes* ainda são escassos, limitando assim nossa capacidade de otimizar a seleção de genes de referência em diferentes tratamentos experimentais (XIANG et al., 2018).

Diante disso, a análise transcriptômica é muito útil, pois permite a comparação de perfis de expressão em diferentes linhagens, estágios de desenvolvimento e condições experimentais. Além disso, a validação das diferenças quantitativas encontradas no sequenciamento através de técnicas complementares, como a RT-qPCR, possibilita a dedução dos níveis de expressão (MEI et al., 2003). Desta forma, uma linhagem com deficiência na formação da capa marrom, deverá apresentar menor expressão de determinados genes em relação à linhagem eficiente na formação da capa marrom.

REFERÊNCIAS

ABRAHÃO, M. C. et al. Ligninolytic activity from newly isolated basidiomycete strains and effect of these enzymes on the azo dye orange II decolourisation. **Annals of Microbiology**, Berlin, v. 58, n. 3, p. 427-432, Sept. 2008.

AGUIAR, A.; FERRAZ, A. Mecanismos envolvidos na biodegradação de materiais lignocelulósicos e aplicações tecnológicas correlatas. **Química Nova**, v. 34, n. 10, p. 1729-1738, 2011.

ALVES, E. et al. Scanning electron microscopy for fungal sample examination. In: **Laboratory Protocols in Fungal Biology**. Springer, New York, NY, p. 133-150, 2013.

BOER, C. G. et al. Purification and some properties of Mn peroxidase from *Lentinula edodes*. **Process Biochemistry**, London, v. 41, n. 5, p. 1203-1207, May 2006.

BRENNAN, M.; LE PORT, G.; GORMLEY, R. Post-harvest treatment with citric acid or hydrogen peroxide to extend the shelf life of fresh sliced mushrooms. **LWT-Food Science and Technology**, v. 33, n. 4, p. 285-289, 2000.

BUSTIN, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. **Journal of molecular endocrinology**, v. 25, n. 2, p. 169-193, 2000.

CANNON, P. F.; KIRK, P. M. **Fungal families of the world**. Wallingford: CABI, p. 456, 2007.

CHANG, S. T.; MILES, P. G**. Mushrooms:** Cultivation, Nutritional Value, Medicinal Effect and Environmental Impact. 2. ed. CRC Press, 2004.

CHEN, X. ***Trichoderma*** **green mold disease on** *Agaricus bisporus*: **Evidence for the introduction of a new genotype of the pathogen.** Ph.D. thesis. The Pennsylvania State University, University Park, 1998.

CHEN, X. et al. PCR-Based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. **Applied and environmental microbiology**, v. 65, n. 6, p. 2674-2678, 1999.

CHEN, A. W.; ARROLD, N.; STAMETS, P. Shiitake cultivation systems. **Mushroom Science**, New York, v. 15, n. 32, p. 771-787, July, 2000.

CHEN, S. C.; GE, W.; BUSWELL, J. A. Molecular cloning of a new laccase from the edible straw mushroom *Volvariella volvacea*: possible involvement in fruit body development. **FEMS Microbiology Letters**, Amsterdam, v. 230, n. 2, p. 171-176, Jan. 2004.

CHEN, A. W. What is shiitake? In: GUSH, R. (Ed.) Shiitake cultivation. Seoul: Mush World, p. 3-32, 2005.

CHUM, W. W. Y. et al. Gene expression studies of the dikaryotic mycelium and primordium of *Lentinula edodes* by serial analysis of gene expression. **Mycological Research**, Cambridge, v. 112, n. 8, p. 950-964, Aug. 2008.

CHUM, W. W. Y. et al. Cataloging and profiling genes expressed in *Lentinula edodes* fruiting body by massive cDNA pyrosequencing and LongSAGE. **Fungal genetics and biology**, v. 48, n. 4, p. 359-369, 2011.

CLAUS, H.; DECKER, H. Bacterial tyrosinases. **Systematic and Applied Microbiology**, Stuttgart, v. 29, n. 1, p. 3-14, 2006.

CLOSE, T. J. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. **Physiologia Plantarum**, v. 97, n. 4, p. 795-803, 1996.

CZECHOWSKI, T. et al. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. **Plant physiology**, v. 139, n. 1, p. 5-17, 2005.

ESPOSITO, E.; CANHOS, V. P.; DURDN, N. Screening of lignin-degrading fungi for removal of color from kraft mill wastewater with no additional extra carbon-source. **Biotechnology Letters**, Dordrecht, v. 13, n. 8, p. 571-576, Aug. 1991.

EXPÓSITO-RODRÍGUEZ, M. et al. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. **BMC plant biology**, v. 8, n. 1, p. 131, 2008.

FERRARONI, M. et al. Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. **BMC Structure Biology**, Bethesda, v. 7, p. 60-61, Sept. 2007.

GINZINGER, D. G. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. **Experimental hematology**, v. 30, n. 6, p. 503-512, 2002.

GLENN, J. K.; GOLD, M. H. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. **Archives of Biochemistry and Biophysics**, New York, v. 242, n. 2, p. 329-341, Nov. 1985.

GOODELL, B. Wood deterioration and preservation advances in our changing world. In: **ACS symposium series**. v. 845, p. 378-398, 2003.

GROGAN, H. M. et al. Control of *Trichoderma harzianum*, a weed mould of mushroom cultivation. 1996.

GROGAN, H. M. et al. Compost inoculation and control of *Trichoderma harzianum*–a weed mold of mushroom cultivation. **Mushroom News**, v. 45, n. 4, p. 29-36, 1997.

HAMILTON, A. J.; GOMEZ, B. L. Melanins in fungal pathogens. **Journal of medical microbiology**, v. 51, n. 3, p. 189, 2002.

HAYWARD-LESTER, A. et al. Accurate and absolute quantitative measurement of gene expression by single-tube RT-PCR and HPLC. **Genome Research**, v. 5, n. 5, p. 494-499, 1995.

HERNÁNDEZ-ORTEGA, A.; FERREIRA, P.; MARTÍNEZ, A. T. Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. **Applied and Environmental Microbiology**, Washington, v. 93, n. 4, p. 1395-1410, Feb. 2012.

HILDEN, K. S. et al. Molecular characterization of the basidiomycete isolate *Nematoloma frowardii* b19 and its manganese peroxidase places the fungus in the corticioid genus *Phlebia*. **Microbiology**, New York, v. 154, n. 8, p. 2371-2379, Aug. 2008.

HOFRICHTER, M. Review: lignina conversion by manganese peroxidase (MnP). **Enzyme and Microbial Technology**, New York, v. 30, n. 4, p. 454-466, Apr. 2002.

HOSHINO, F. et al. Thermally stable and hydrogen peroxide tolerant manganese peroxidase (MnP) from *Lenzites betulinus*. **FEBS Letters**, Amsterdam, v. 530, n. 1, p. 249-252, Oct. 2002.

HOWELL, C. R. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. **Plant disease**, v. 87, n. 1, p. 4-10, 2003.

ISHIKAWA, N. K.; KASUYA, M. C. M.; VANETTI, M. C. D. Antibacterial activity of *Lentinula edodes* grown in liquid medium. **Brazilian Journal of Microbiology**, São Paulo, v. 32, n. 3, p. 206-210, 2001.

JIANG, T.; LUO, Z.; YING, T. Fumigation with essential oils improves sensory quality and enhanced antioxidant ability of shiitake mushroom (*Lentinus edodes*). **Food chemistry**, v. 172, p. 692-698, 2015.

JOHANSSON, T.; WELINDER, K. G.; NYMAN, P. O. Isozymes of lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*: II., partial sequences, peptide maps, and amino acid and carbohydrate compositions. **Archives of Biochemistry and Biophysics**, New York, v. 300, n. 1, p. 57-62, Jan. 1993.

KALBERER, P. P. Influenece of urea and ammonium chloride on crop yield and fruit body size of shiitake (*Lentinula edodes*). **Mushroom Science**, New York, v. 15, n. 32, p. 361-366, July, 2000.

KANDA, K. et al. Purification and properties of tyrosinase isozymes from the gill of *Lentinus edodes* fruiting body. **Bioscience, Biotechnology, and Biochemistry**, Tokyo, v. 60, n. 8, p. 1273-1278, 1996.

KIRK, T. K.; CULLEN, D. Enzymology and molecular genetics of wood degradation by white-rot fungi. **Environmentally friendly technologies for the pulp and paper industry.** Wiley, New York, p. 273-307, 1998.

KLEIN, C. A. et al. Combined transcriptome and genome analysis of single micrometastatic cells. **Nature biotechnology**, v. 20, n. 4, p. 387, 2002.

LANGFELDER, K. et al. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. **Fungal Genetics and Biology**, Orlando, v. 38, n. 2, p. 143-158, 2003.

LANKINEN, V. P. et al. Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of *Agaricus bisporus*. **Applied and Environmental Microbiology**, Washington, v. 55, n. 2, p. 170-176, Mar. 2001.

LAZAROTTO, M. et al. Identification of mold mungi in pinus wood under laboratory conditions. **Floresta e Ambiente**, v. 23, n. 4, p. 602-605, 2016.

LEATHAM, G. F.; STAHMANN, M. A. Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. **Journal of General Microbiology**, London, v. 125, p. 147-157, 1981.

LEE, S. S. et al. Several genes expressed during morphogenesis of *Lentinus edodes* (ImHyup-1). **Mycobiology**, v. 29, n. 3, p. 135-141, 2001.

LEE, H. M. et al. Breeding and screening of *Lentinula edodes* strains resistant to *Trichoderma* sp. **Mycobiology**, v. 36, n. 4, p. 270-273, 2008.

LI, H. et al. De novo transcriptome of safflower and the identification of putative genes for oleosin and the biosynthesis of flavonoids. **PloS one**, v. 7, n. 2, p. e30987, 2012.

LISOV, A. V.; LEONTIEVSKY, A. A.; GOLOVLEVA, L. A. Hybrid Mn-peroxidase from the ligninolytic fungus *Panus tigrinus* 8/18: isolation, substrate specificity and catalytic cycle. **Biochemistry**, Moscow, v. 68, n. 9, p. 1027-1035, Sept. 2003.

MARQUES, P. R. B. O.; YAMANAKA, H. Biossensores baseados no processo de inibição enzimática. **Química Nova**, São Paulo, v. 31, n. 7, p. 1791-1799, 2008.

MARTÍNEZ, A. T. et al. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. **International Microbiology**, Madrid, v. 8, n. 3, p. 195-204, Sept. 2005.

MEI, R. et al. Probe selection for high-density oligonucleotide arrays. **Proceedings of the National Academy of Sciences**, v. 100, n. 20, p. 11237-11242, 2003.

MENOLLI JUNIOR, N.; PACCOLA-MEIRELLES, L. D. Obtention of *Lentinula edodes* strains resistant to high temperatures and selection of strains resistant to *Trichoderma* sp. **Ciência e Agrotecnologia**, Lavras, v. 34, p. 1640-1646, dez. 2010. Edição especial.

MEYERS, B. C. et al. Methods for transcriptional profiling in plants. Be fruitful and replicate. **Plant Physiology**, v. 135, n. 2, p. 637-652, 2004.

MINHONI, M. T. A. et al. **Cultivo de *Lentinula edodes* (Berk.) Pegler - (Shiitake).** 3. ed. Botucatu: FEPAF, 2007.

MITRA, R. D. et al. Fluorescent in situ sequencing on polymerase colonies. **Analytical biochemistry**, v. 320, n. 1, p. 55-65, 2003.

MIYAZAKI, Y.; NAKAMURA, M.; BABASAKI, K. Molecular cloning of developmentally specific genes by representational difference analysis during the fruiting body formation in the basidiomycete *Lentinula edodes*. **Fungal Genetics and Biology**, v. 42, n. 6, p. 493-505, 2005.

MODA, E. M. et al. Uso de peróxido de hidrogênio e ácido cítrico na conservação de cogumelo *Pleurotus sajor-caju* in natura. **Ciência e Tecnologia de Alimentos**, v. 25, n. 2, 2005.

NAGAI, M. et al. Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. **Microbiology**, New York, v. 149, n. 9, p. 2455-2462, 2003.

NAGALAKSHMI, U. et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. **Science**, v. 320, n. 5881, p. 1344-1349, 2008.

NAKAMURA, M. et al. Identification of copper ligands in *Aspergillus oryzae* tyrosinase by site-directed mutagenesis. **Biochemical Journal**, London, v. 350, n. 2, p. 537-545, 2000.

NICOT, N. et al. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. **Journal of experimental botany**, v. 56, n. 421, p. 2907-2914, 2005.

OHGA, S. et al. Transcriptional regulation of laccase and cellulase in relation to fruit body formation in the mycelium of *Lentinula edodes* on a sawdust-based substrate. **Mycoscience**, Tokyo, v. 41, n. 2, p. 149-153, Apr. 2000.

OSPINA-GIRALDO, M. D. et al. Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* sp. associated with mushroom green mold. **Phytopathology**, v. 89, n. 4, p. 308-313, 1999.

PAOLA, G. Laccases: a never-ending story. **Cellular and Molecular Life Sciences**, Basel, v. 67, n. 3, p. 369-385, Feb. 2010.

POZDNIAKOVA, N. N. et al. Yellow laccase from the fungus *Pleurotus ostreatus* D1: purification and characterization. **Applied Biochemistry and microbiology**, Moscow, v. 42, n. 1, p. 56-61, 2006.

RENGANATHAN, V.; MIKI, K.; GOLD, M. H. Multiple molecular forms of diarylpropane oxygenase, an H_2O_2-requiring, lignin-degrading enzyme from *Phanerochaete chrysosporium*. **Archives of Biochemistry and Biophysics**, New York, v. 241, n. 1, p. 304-314, Aug. 1985.

ROCHA, L. M.; MOREIRA, L. M. A. Diagnóstico laboratorial do albinismo oculocutâneo. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, Rio de Janeiro, v. 43, n. 1, p. 25-30, 2007.

RODGERS, C. J. et al. Designer laccases: a vogue for high-potential fungal enzymes. **Trends in Biotechnology**, Amsterdam, v. 28, n. 2, p. 63-72, Feb. 2010.

ROYSE, D. J. Cultivation of shiitake on natural and synthetic logs. **College of Agricultural Sciences, Agricultural Research and Cooperative Extension. The Pennsylvania State University.** 2009.

ROYSE, D. J.; BAARS, J.; TAN, Q. Current overview of mushroom production in the world. **Edible and medicinal mushrooms:** technology and applications, p. 5-13, 2017.

DE LA RUBIA, T. et al. Characterization of manganese-dependent peroxidase isoenzymes from the ligninolytic fungus *Phanerochaete flavido-alba*. **Research in Microbiology**, Paris, v. 153, n. 8, p. 547-554, Oct. 2002.

RUIZ-DUEÑAS, F. J.; MARTÍNEZ, A. T. Structural and functional features of peroxidases with a potential as industrial biocatalysts. In: **Biocatalysis based on heme peroxidases**. Springer, Berlin, Heidelberg, p. 37-59, 2010.

SAKAMOTO, Y. et al. Cloning of *Lentinula edodes* lemnp2, a manganese peroxidase that is secreted abundantly in sawdust medium. **Mycoscience**, Tokyo, v. 50, n. 2, p. 116-122, Mar. 2009.

SAKAMOTO, Y.; NAKADE, K.; SATO, T. Characterization of the post-harvest changes in gene transcription in the gill of the *Lentinula edodes* fruiting body. **Current genetics**, v. 55, n. 4, p. 409, 2009.

SAPERS, G. M. et al. Shelf-life extension of fresh mushrooms (*Agaricus bisporus*) by application of hydrogen peroxide and browning inhibitors. **Journal of Food Science**, v. 66, n. 2, p. 362-366, 2001.

SEABY, D. *Trichoderma* as a weed mould or pathogen in mushroom cultivation. **Trichoderma and Gliocladium**, v. 2, p 267-272, 1998.

SHLEEV, S. et al. Characterization of two new multiforms of *Trametes pubescens* laccase. **Bioorganic Chemistry**, New York, v. 35, n. 1, p. 35-49, Feb. 2007.

STEPHEN, B. et al. Screening of basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific sequences. **Mycological Research**, v. 109, n. 1, p. 115-124, 2005

STURM, R. A.; TEASDALE, R. D.; BOX, N. F. Human pigmentation genes: identification, structure and consequences of polymorphic variation. **Gene**, v. 277, n. 1-2, p. 49-62, 2001.

SUIZU, T. et al. Analysis of expressed sequence tags (ESTs) from *Lentinula edodes*. **Applied microbiology and biotechnology**, v. 79, n. 3, p. 461-470, 2008.

TAKANO, M.; NAKAMURA, M.; YAMAGUCHI, M. Glyoxal oxidase supplies hydrogen peroxide at hyphal tips and on hyphal wall to manganese peroxidase of white-rot fungus *Phanerochaete crassa* WD1694. **Journal of Wood Science**, London, v. 56, n. 4, p. 307-313, 2010.

TANG, L. et al. Transcriptome analysis of candidate genes and signaling pathways associated with light-induced brown film formation in *Lentinula edodes*. **Applied Microbiology & Biotechnology**, Berlin, v. 97, n. 11, p. 4977-4989, June 2013.

TERASHIMA, Y.; IGUSA, H.; OHGA, S. Influence of contamination by *Penicillium brevicompactum* and *Trichoderma harzianum* during *Lentinula edodes* Spawn run on fruiting in sawdust-based substrates. **Journal Mycoscience**, Tokyo, v. 34, n. 54, p. 277-280, Feb. 2002.

TIEN, M.; KIRK, T. K. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. **Proceedings of the National Academy of Science of the United States of America**, Washington, v. 81, n. 8, p. 2280-2284, Apr. 1984.

TOKIMOTO, K. Physiological studies on antagonism between *Lentinula edodes* and *Trichoderma* sp. in bedlogs of the former (in Japanese). **Reports of the Tottori Mycological Institute**, v. 23, p. 1-54, 1985.

TSIVILEVA, O. M. et al. Effect of media components on the mycelial film formation in submerged culture of *Lentinus edodes* (Shiitake). **Food Technology and Biotechnology**, Zagreg, v. 43, n. 3, p. 227-234, 2005.

ULHOA, C. J.; PEBERDY, J. F. Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. **Enzyme and Microbial Technology**, v. 14, n. 3, p. 236-240, 1992.

URBEN, A. F. Produção de cogumelos por meio de tecnologia chinesa modificada. **Brasília: Embrapa Recursos Genéticos e Desenvolvimento**, 2004.

XIANG, Q. et al. Identification and evaluation of reference genes for qRT-PCR studies in *Lentinula edodes*. **PloS one**, v. 13, n. 1, p. e0190226, 2018.

XU, M. et al. Reference gene selection for quantitative real-time polymerase chain reaction in Populus. **Analytical biochemistry**, v. 408, n. 2, p. 337-339, 2011.

WAN, H. et al. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. **Analytical biochemistry**, v. 399, n. 2, p. 257-261, 2010.

WANG, N.; HEBERT, D. N. Tyrosinase maturation through the mammalian secretary pathyway: bringing color to life. **Pigment Cell Research**, London, v. 19, n. 1, p. 3-18, 2006.

WANG, B. et al. Survey of the transcriptome of *Aspergillus oryzae* via massively parallel mRNA sequencing. **Nucleic acids research**, v. 38, n. 15, p. 5075-5087, 2010.

WARIISHI, H.; AKILESWARAN, L.; GOLD, M. H. Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. **Biochemistry**, v. 27, n. 14, p. 5365-5370, 1988.

WICHERS, H. J. et al. Cloning, expression, and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. **Applied Microbiology and Biotechnology**, Oxford, v. 61, n. 4, p. 336-341, 2003.

WONG, D. W. Structure and action mechanism of ligninolytic enzymes. **Applied Microbiology and Biotechnology**, Berlin, v. 157, n. 2, p. 174-209, 2009.

WONG, M. M. L.; CANNON, C. H.; WICKNESWARI, R. Identification of lignin genes and regulatory sequences involved in secondary cell wall formation in *Acacia auriculiformis* and *Acacia mangium* via de novo transcriptome sequencing. **BMC genomics**, v. 12, n. 1, p. 342, 2011.

YOU, Y. et al. Selection of reliable reference genes for quantitative real-time PCR analysis in plum (*Prunus salicina* Lindl.) under different postharvest treatments. **Scientia Horticulturae**, v. 210, p. 285-293, 2016.

YU, J. et al. Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq. **FEMS microbiology letters**, v. 322, n. 2, p. 145-149, 2011.

ZHANXI, L.; ZHANHUA, L. Jun-cao technology. **China Agricultural Scientech press, Beijing**, p. 252, 2001.

SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Enzymatic activity during shiitake brown film production

Artigo será submetido de acordo com as normas da revista Journal of Agricultural and Food Chemistry (Impact factor: 3.412)

Abstract

Brown film formation is a step that precedes the primordia initiation in *Lentinula edodes* and has an important role performing a protective function. In addition, being a living, metabolically active, and multifunctional dynamic system performs important regulatory functions. Therefore, the enzymatic activity is closely related to brown film formation and consequently in the formation and development of fruiting bodies. Thus, the present work intended to evaluate the ligninases and tyrosinase activities of two shiitake strains, UFLA-LE5 (less productive) and UFLA-LE6 (productive), in three sampling with the purpose of better understand these enzymes action in the brown film formation. The laccase activity was the lowest and decreased continuously for all strains. Manganese peroxidase activity increased in the internal samples, while in the external samples the values were decreasing over time, for both strains. On the other hand, lignin peroxidase activity was much higher when compared to other enzymes and remained relatively stable. The enzymatic production of tyrosinase was quite varied for the two strains, the UFLA-LE5 showed a peak of this enzyme at 60 days while UFLA-LE6 already started with a high activity. This may explain the fact that UFLA-LE6 forms the brown film faster than UFLA-LE5, which needs more time to reach the enzyme peak.

Keywords: *Lentinula edodes*; ligninases; tyrosinase.

Introduction

The worldwide production of *Lentinula edodes* (Berk.) Pegler, also known as shiitake, has increased in the latest years and it is one of the most popular fungus cultivated (CHANG; WASSER, 2017). The shiitake mushroom is highly nutritive, with low calories and high amounts of vitamins, proteins and minerals. Besides, they contain some elements essential to human nutrition such as calcium, copper, phosphorus, manganese, magnesium and zinc (EMERY, 2005; KALAČ, 2013). It was also pointed out that mushrooms possess medicinal properties such as anti-inflammatory, anti-tumor,

antibacterial and antioxidant (GARCÍA-LAFUENTE et al., 2010; PATEL; GOYAL, 2012; VALVERDE; HERNÁNDEZ-PÉREZ; PAREDES-LÓPEZ, 2015).

Shiitake is a wood-decomposer fungus able to use lignin, cellulose and hemicellulose as carbon sources. Due to such skills, its traditional cultivation is done on logs of various species of trees such as oak and eucalyptus (ZHANXI; ZHANHUA, 2001). However, the axenic cultivation based upon different agricultural residues like sawdust, has been gaining popularity in commercial production because harvesting time is lower and the productivity is higher (PHILIPPOUSSIS; DIAMANTOPOULOU; ISRAILIDES, 2007; ZIED et al., 2016).

According to Tang et al. (2013), the cultivation cycle of shiitake can be divided into four stages: vegetative mycelial growth, browning process, primordium formation and fruiting body development. The growth phases are affected by the proportion of lignin and cellulose as well as by the nitrogen content of the substrate. As a white rot fungus, *L. edodes* secretes important oxidoreductases including lignin peroxidase (1,2-bis (3,4-dimethoxylphenyl) propene-1,3- diol:hydrogen-peroxide), manganese peroxidase (Mn(II):hydrogen-peroxide oxidoreductase) and laccase (benzenediol:oxygen reductase) (PASZCZYNSKI; HUYNH; CRAWFORD, 1986; GLENN; AKILESWARAN; GOLD, 1986; MAYER; HAREL, 1979). These enzymes oxidise, in a nonspecific way, both phenolic and non-phenolic lignin derivatives (FAHR et al., 1999; FERREIRA-LEITÃO; DE CARVALHO; BON, 2007).

After the substrate is colonized, the non pigmented vegetative mycelium become denser and acquires pigmentation. The vegetative cells eventually become coated with a layer of interwoven stained hyphae and a brown mycelial layer is produced (VETCHINKINA; NIKITINA, 2007). This step precedes the appearance of the fruiting bodies and has been described as an essential step in the morphogenesis for shiitake (CHUM et al., 2008; TSIVILEVA et al., 2005). However, the molecular and biochemical mechanisms of its formation are still unclear (TANG et al., 2013).

There are a lot of studies about morphological, physiological and biochemical features of a number of *L. edodes* strains at various stages of their development. For a long time, hardly any studies have investigated this cell surface structure, because it had believed that it performs only a “skeletal” protective function against mycelium dehydration (GARIBOVA et al., 2009). However, it was observed that certain strains exhibited some specific features: after substrate colonization and mycelial packing, shapeless hypertrophied primordia formed, and normal fruiting did not occur. According Vetchinkina and Nikitina (2007), some shiitake strains that did not form a brown cover were also incapable of fruiting. In the same context, Vetchinkina et al. (2015) also relateded that after the brown mycelial layer began to develop, normal primordia and fruiting bodies appeared.

Then, in our study we selected the strains UFLA-LE5 and UFLA-LE6, which present a different profile in the brown film formation. Strain UFLA-LE6 forms the brown film rapidly and has a very high

productivity rate; while the UFLA-LE5 strain, presents a slow in the brown film formation and consequently a low productivity of mushrooms (SOUSA et al., 2018). Another interesting fact is that some studies already pointed out the relation between enzyme activity and the brown film formation, but they have been conducted only in synthetic culture media, not under the real cultivation conditions of shiitake (TSIVILEVA et al., 2005; VETCHINKINA; SOKOLOV; NIKITINA, 2008). Therefore, it is important to evaluate the activity of potential candidate proteins using different strains with diverse levels of productivity in a standard cultivation system.

Thus, the present work aimed to determine the ligninases and tyrosinase activities of two strains of *Lentinula edodes* in different times of the brown film formation in order to better understand the enzymes roles possibly involved in this process.

Materials and methods

Microrganisms and culture conditions

In the experiment, two strains of *Lentinula edodes*, UFLA-LE5 (less productive) and UFLA-LE6 (productive) as described by Zied et al. (2016) were utilized. The stock cultures were kept in potato dextrose agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g; and deionized water, 800 ml) and incubated at 25ºC for 7 days.

Spawn preparation

The spawn was prepared using *Eucaliptus* sawdust (80%), wheat meal (20%), gypsum (2%) and limestone (2%). The moisture was adjusted to 60% and 200g of that substrate were distributed into glass flasks, which were autoclaved twice for two hours at 121°C with a 24-hours interval. After inoculation of the strains, the flasks were incubated at room temperature (25 ± 2°C) until the substrate was completely colonized by the fungus.

Axenic cultivation

The substrate for axenic cultivation of the strains of *L. edodes* was prepared utilizing *Eucalyptus* sawdust (25 kg); rice bran (2 kg); wheat bran (0.3 kg); cottonseed meal (0.3 kg); coarse cornmeal (0.3 kg) and calcium carbonate (0.06 kg). The moisture was adjusted to 60% and 2 kg of substrate was placed in a high density polyethylene bag with a 4 cm^2 filter. The bags were sealed and two autoclaving processes of 2 hours with a 24-hours interval were carried out. After cooling to room temperature, the substrates were inoculated with 3% spawn and incubated at room temperature (25 ± 2ºC) for the colonization and browning process.

Sample collection

Substrate samples were collected at three times in order to follow the entire brown film formation period. At each sampling time, three blocks were utilized from each strain. Random samples were taken from the outside and inside the block, which the blocks were completely broken up. The first sample was obtained 30 days after inoculation, when the blocks were already fully colonized. After 60 days the second sample was removed and the third sample was got at 90 days, when the browning process was considered complete. The activities of tyrosinase and ligninases were determined for each sample collected.

Preparation of crude protein extract

The crude extract for tyrosinase and ligninases analyses was prepared according to Luz et al. (2012), with modifications. The samples were macerated and 20 g were placed in an Erlenmeyer (250 mL) with 100 mL of sodium citrate buffer (50 mmol L^{-1}, pH 4.8), shaken at 150 rpm for 2 h and filtered utilizing filter paper (Whatman n. 1). All enzyme assays were conducted in triplicate.

Enzyme assays

Laccase (EC 1.10.3.2)

Laccase activity was determined by indirect spectrophotometric method utilizing 2,2-azino-bis ethylbenthiazoline (ABTS) in reaction mixture of 1mL containing 0.3 mL of sodium acetate 0.1 mol L^{-1} buffer (pH 5,0); 0.1 mL of ABTS 1 mmol L^{-1} (in water) and 0.6 mL of the enzymatic source. The reaction mixture was incubated for 10 minutes at 37ºC and the ABTS oxidation was measured by the increase of absorbance at 420 nm (BUSWELL; CAI; CHANG, 1995). A unit of enzymatic activity was defined as the amount of enzyme capable of oxidizing 1µmol of ABST per minute (ε_{420} = 3.6 x 10^4 mol^{-1} L cm^{-1}), per kg.

Manganese peroxidase (EC 1.11.1.13)

Described by Kuwahara et al. (1984), the activity of manganese peroxidase was measured utilizing phenol red (1 g L^{-1}) as a substrate (ε_{610} = 4.460 mol^{-1} L cm^{-1}). The absorbance was measured at 610 nm and the unit of enzyme activity was defined as the amount of enzyme capable of oxidizing 1 µmol of phenol red per minute per kg.

Lignin peroxidase (EC 1.11.1.14)

The lignin peroxidase activity was determined by the monitoring of absorbance at 310 nm of the formation of veratraldehyde (ε_{310} = 9,300 mol^{-1} L cm^{-1}), by means of the oxidation of veratryl alcohol

(TIEN; KIRK, 1984). A unit of enzyme activity was defined as the amount of enzyme capable of oxidizing 1 µmol of veratryl acid per minute per kg.

Tyrosinase (E.C.1.14.18.1)

Determined by the oxidation rate of L-di-hydroxiphenylalanine (L-DOPA) 2 mmol L^{-1} in 50 mmol L^{-1} Tris-HCl buffer (pH 7.5) at 37 ºC for 10 minutes. The oxidation of L-DOPA was measured according to Pomerantz and Murthy (1974) by the increase of absorbance at 475 nm (ε_{475} 3700 $M^{-1}cm^{-1}$). A unit of enzyme activity was defined as the amount of enzyme capable of oxidizing 1 µmol of L-DOPA per minute per kg.

Statistical analysis

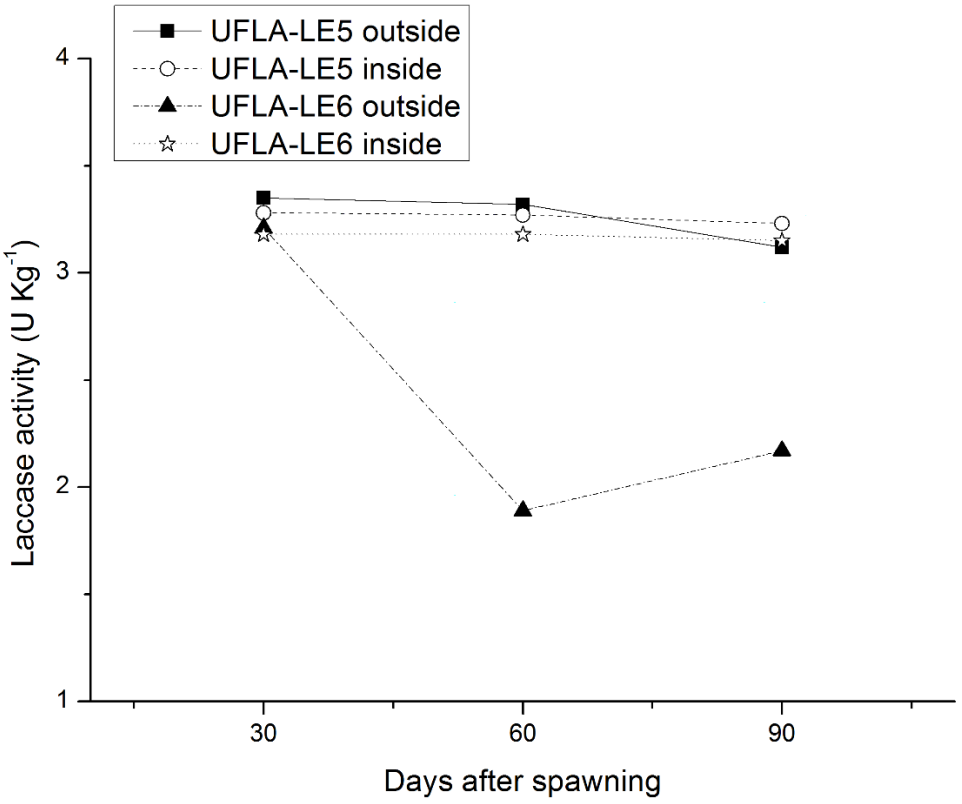
The experiment was arranged in a completely randomized design (DRC) with factorial scheme (time x strains). Data were submitted to the analysis of variance and the Tukey test was used to compare the means ($P<0.05$) using SISVAR software (FERREIRA, 2008).

Results and Discussion

Laccase activity for UFLA-LE5 and UFLA-LE6 strains was higher in the samples from outside region at 30 days (3.35 and 3.21 U kg^{-1}, respectively). At 90 days, the situation reversed and the inside samples showed a greater activity. The two strains presented a profile in which the enzymatic activity decreases over time, however the outside sample of UFLA-LE6 strain shows a slight increase in the value at 90 days (Figure 1). This may indicate an increase in the activity of this strain, especially when brown film is well established, a fact evidenced by Sousa et al. (2018), where UFLA-LE6 strain presented the highest laccase activity at 159 days (20.18 U kg^{-1}). In *Agaricus bisporus* and *L. edodes*, the laccase activity is reported to be higher immediately before the formation of the fruiting bodies and declining rapidly after formation of primordia (KÜES; LIU, 2000). In addition, laccase-negative mutants of *Pleurotus ostreatus* (cv. Florida) did not form fruiting bodies (VETCHINKINA et al., 2017).

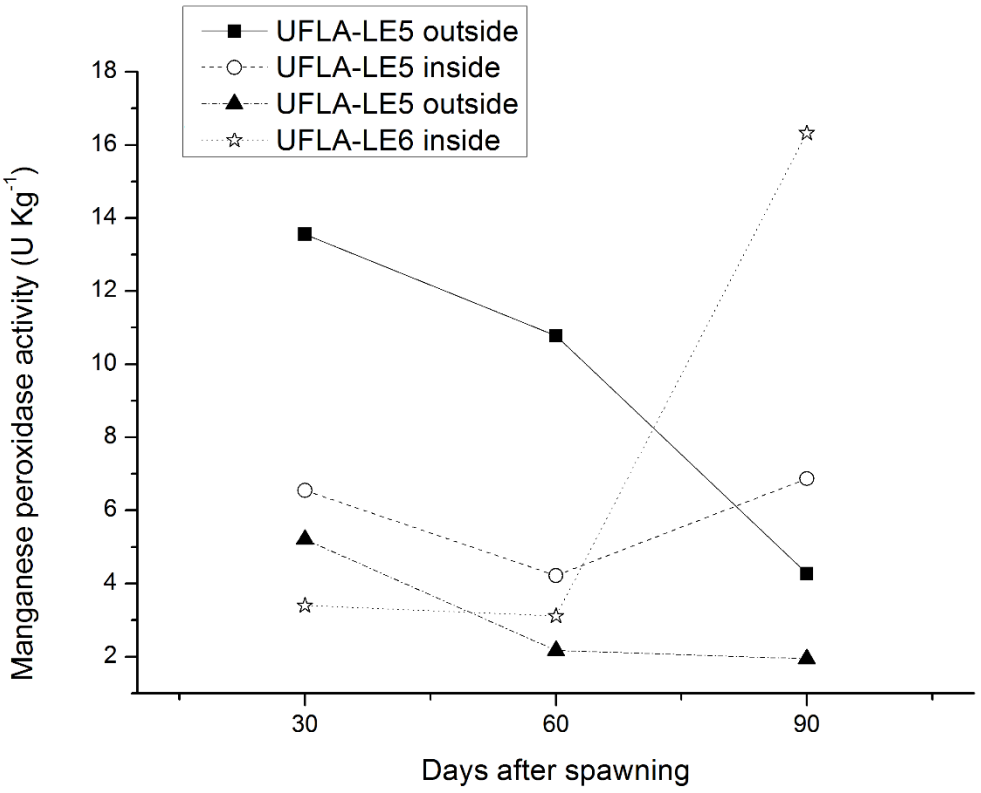
In *L. edodes* the browning process is accelerated by oxygen and laccase activity, as a result of the formation of phenolic compounds, water droplets are produced because oxygen is reduced to water (ARORA; SHARMA, 2010; TANG et al., 2013). This explains why large quantities of water are observed in the plastic bag during the browning process, necessitating that the water be drained. Although, the laccase possesses a great deal of beneficial qualities, after mushroom harvest, an increase in this enzyme activity could cause its browning, resulting in a short shelf life, since the mushroom appearance becomes disagreeable (YE et al., 2012).

Figure 1 - Laccase activity (U: unit of enzymatic activity) of samples from outside and inside of the substrate of UFLA-LE5 and UFLA-LE6 *Lentinula edodes* strains in different times.



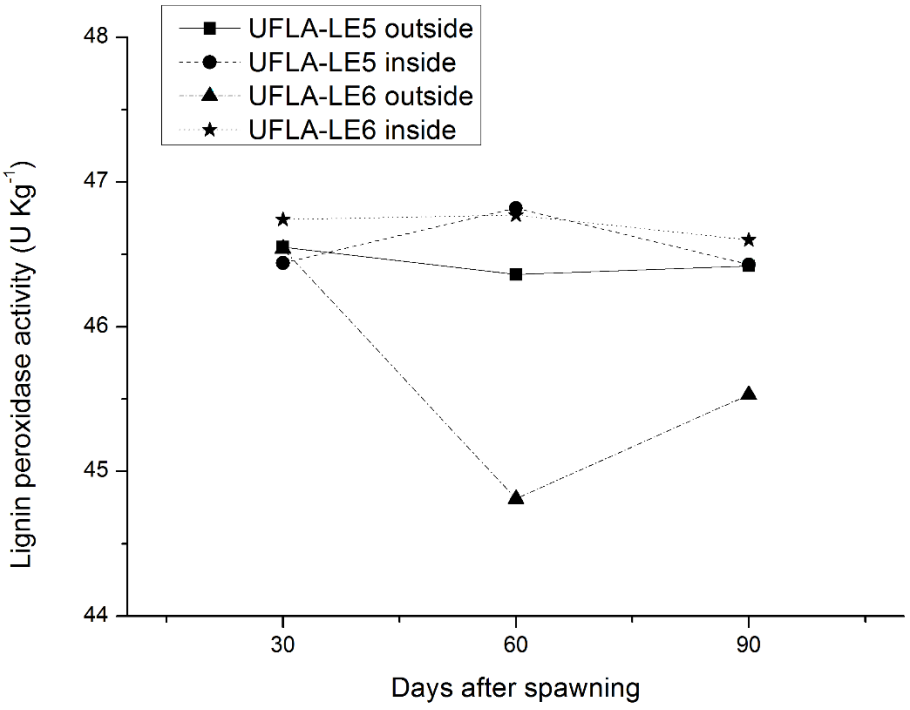
There was a general decline in manganese peroxidase activity in outside samples over the three sampling except of inside samples that showed an increased activity at 90 days. Comparing the outside samples, the UFLA-LE5 strain presented an initial activity of 13.56 U kg^{-1} against 5.21 U kg^{-1} of the UFLA-LE6 strain (Figure 2).

Figure 2 - Manganese peroxidase activity (U: unit of enzymatic activity) of samples from outside and inside of the substrate of UFLA-LE5 and UFLA-LE6 *Lentinula edodes* strains in different times.



Lignin peroxidase activity was substantially higher when compared to other enzymes, presenting the highest initial activity of 46.74 U kg^{-1} and highest final activity of 46.60 U kg^{-1}, both for UFLA-LE6 strain. During the period between 60 and 90 days, the lignin peroxidase increased in the outside samples and decreased in the inside samples, however, the enzyme activity was slightly higher in the inside samples (Figure 3).

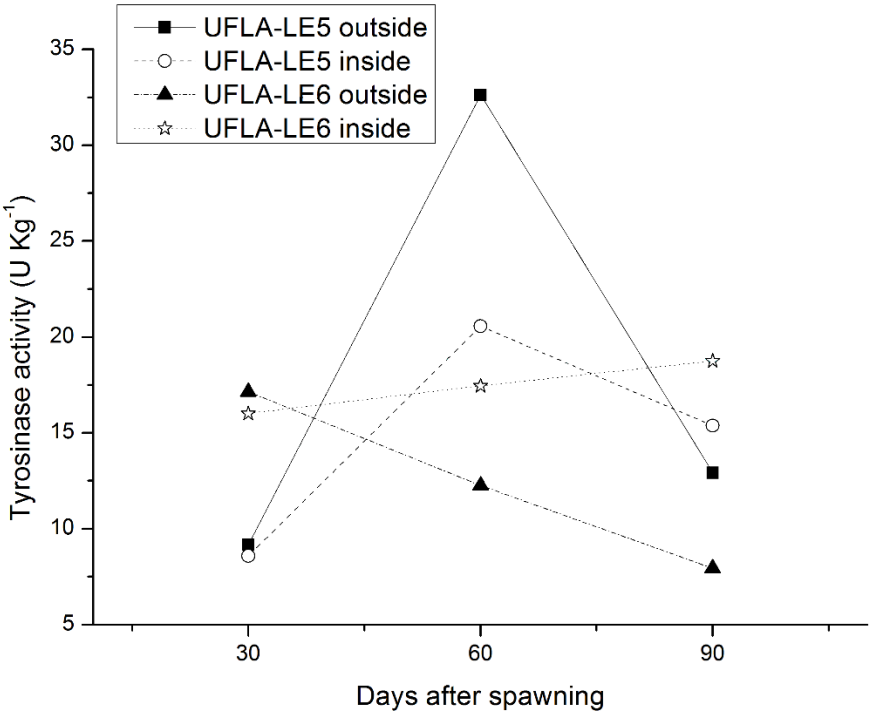
Figure 3 - Lignin peroxidase activity (U: unit of enzymatic activity) of samples from outside and inside of the substrate of UFLA-LE5 and UFLA-LE6 *Lentinula edodes* strains in different times.



In our study, the laccase and manganese peroxidase activities were very lower compared to the activity of lignin peroxidase for the two strains and in all sampling periods. According to Martínez (2002), when the activities of these two enzymes are lower, the activity of lignin peroxidase is higher. This substantially higher activity is an evidence that lignin peroxidase, due to their high redox potential, plays the main function in lignin degradation and consequently in brown film formation (GOLD; YOUNGS; GELPKE, 2000).

The tyrosinase activity of the outside samples reveals different behaviors of the two *L. edodes* strains. Since the UFLA-LE5 strain shows a peak of enzymatic activity at 60 days (32.62 U kg^{-1}) and at 90 days it decreases (12.92 U kg^{-1}), whereas the UFLA-LE6 strain begins with a high activity (17.15 U kg^{-1}) and then decreases over time (7.94 U kg^{-1}) (Figure 4). These results may explain the fact that UFLA-LE6 strain forms the brown cover faster and has higher productivity indexes (SOUSA et al., 2018).

Figure 4 - Tyrosinase activity (U: unit of enzymatic activity) of samples from outside and inside of the substrate of UFLA-LE5 and UFLA-LE6 *Lentinula edodes* strains in different times.



Tyrosinase activity was much higher compared to laccase activity under all conditions of the experiment. In contrast to manganese peroxidase and lignin peroxidase, the low redox potential of tyrosinase suggests that this enzyme does not take part in degradation processes, but in the pigment production during the browning process (WICHERS et al., 2003). This agrees with some published data that indicate a link between the increase in tyrosinase activity and brown film synthesis, since this enzyme is found in the metabolic pathway of melanin synthesis (VETCHINKINA et al., 2017). Its high activity from the beginning of substrate colonization is evidence for its importance thorough out all the cultivation cycles.

In conclusion, considering all parameters evaluated in this work with the use of different strains with different profiles, we may state that brown film formation is more complex than the factors that were measured. Although our results may be useful for isolating from the brown film highly active proteins, pigments and other biologically active compounds of industrial importance. Therefore, more detailed studies are necessary to establish other factors that not only induce this process but other metabolic processes related to substrate degradation, biosynthesis reactions and mushroom production.

Acknowledgements

The FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) are acknowledged for financial support.

References

ARORA, D. S.; SHARMA, R. K. Ligninolytic fungal laccases and their biotechnological applications. **Applied biochemistry and biotechnology**, v. 160, n. 6, p. 1760-1788, 2010.

BUSWELL, J. A.; CAI, Y; CHANG, S. T. Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinula* (Lentinus) *edodes*. **FEMS microbiology letters**, v. 128, n. 1, p. 81-87, 1995.

CHANG, S.; WASSER, S. The cultivation and environmental impact of mushrooms. 2017.

CHUM, W. W. Y. et al. Gene expression studies of the dikaryotic mycelium and primordium of *Lentinula edodes* by serial analysis of gene expression. **Mycological research**, v. 112, n. 8, p. 950-964, 2008.

EMERY, P. W. Metabolic changes in malnutrition. **Eye**, v. 19, n. 10, p. 1029-1034, 2005.

FAHR, K. et al. Degradation of 2, 4-dichlorophenol and pentachlorophenol by two brown rot fungi. **FEMS Microbiology Letters**, v. 175, n. 1, p. 127-132, 1999.

FERREIRA, D. F. SISVAR: um programa para análises e ensino de estatística. **Revista symposium,** p. 36-41, 2008.

FERREIRA-LEITAO, V. S.; DE CARVALHO, M. E. A.; BON, E. P. S. Lignin peroxidase efficiency for methylene blue decolouration: comparison to reported methods. **Dyes and Pigments**, v. 74, n. 1, p. 230-236, 2007.

GARCIA-LAFUENTEA, A. et al. Mushrooms as a source of anti-inflammatory agents. **Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Inflammatory and Anti-Allergy Agents)**, v. 9, n. 2, p. 125-141, 2010.

GARIBOVA, L. V. et al. Biology of *Lentinus edodes*. I. Morphological-cultural and physiological-biochemical peculiarities. **Mikologiya i fitopatologiya**, v. 33, n. 2, p. 107-110, 1999.

GLENN, J. K.; AKILESWARAN, L.; GOLD, M. H. Mn (II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. **Archives of Biochemistry and Biophysics**, v. 251, n. 2, p. 688-696, 1986.

GOLD, M. H.; YOUNGS, H. L.; GELPKE, M. D. Manganese peroxidase. **Metal ions in biological systems**, v. 37, p. 559, 2000.

KALAČ, P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. **Journal of the Science of Food and Agriculture**, v. 93, n. 2, p. 209-218, 2013.

KÜES, U.; LIU, Y. Fruiting body production in basidiomycetes. **Applied microbiology and biotechnology**, v. 54, n. 2, p. 141-152, 2000.

KUWAHARA, M. et al. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. **FEBS letters**, v. 169, n. 2, p. 247-250, 1984.

LUZ, J. M. R. et al. Lignocellulolytic enzyme production of *Pleurotus ostreatus* growth in agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 43, n. 4, p. 1508-1515, 2012

MARTÍNEZ, A. T. Molecular biology and structure-function of lignin-degrading heme peroxidases. **Enzyme and Microbial Technology**, v. 30, n. 4, p. 425-444, 2002.

MAYER, A. M.; HAREL, E. Polyphenol oxidases in plants. P**hytochemistry**, v. 18, n. 2, p. 193-215, 1979.

PASZCZYŃSKI, A.; HUYNH, V. B; CRAWFORD, R. Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. **Archives of Biochemistry and Biophysics**, v. 244, n. 2, p. 750-765, 1986.

PATEL, S.; GOYAL, A. Recent developments in mushrooms as anti-cancer therapeutics: a review. **3 Biotech**, v. 2, n. 1, p. 1-15, 2012.

PHILIPPOUSSIS, A.; DIAMANTOPOULOU, P.; ISRAILIDES, C. Productivity of agricultural residues used for the cultivation of the medicinal fungus *Lentinula edodes*. **International Biodeterioration & Biodegradation**, v. 59, n. 3, p. 216-219, 2007.

POMERANTZ, S. H.; MURTHY, V. V. Purification and properties of tyrosinases from *Vibrio tyrosinaticus*. **Archives of biochemistry and biophysics**, v. 160, n. 1, p. 73-82, 1974.

SOUSA, M. A. D. C. et al. Enzyme activity and biochemical changes during production of *Lentinula edodes* (Berk.) Pegler. **Food Science and Technology**, n. AHEAD, 2018.

TANG, L. H. et al. Transcriptome analysis of candidate genes and signaling pathways associated with light-induced brown film formation in *Lentinula edodes*. **Applied microbiology and biotechnology**, v. 97, n. 11, p. 4977-4989, 2013.

TIEN, M.; KIRK, T. K. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. **Proceedings of the National Academy of Sciences**, v. 81, n. 8, p. 2280-2284, 1984.

TSIVILEVA, O. M. et al. Effect of media components on the mycelial film formation in submerged culture of *Lentinus edodes* (Shiitake). **Food Technology and Biotechnology**, v. 43, n. 3, p. 227-234, 2005.

VALVERDE, M. E.; HERNÁNDEZ-PÉREZ, T.; PAREDES-LÓPEZ, O. Edible mushrooms: improving human health and promoting quality life. **International journal of microbiology**, v. 2015, 2015.

VETCHINKINA, E. P.; NIKITINA, V. E. Morphological patterns of mycelial growth and fruition of some strains of an edible xylotrophic basidiomycete *Lentinus edodes*. **Izv. Samar. Nauch. Tsentr. Ross. Akad. Sci**, v. 9, p. 1085-1090, 2007.

VETCHINKINA, E. P.; SOKOLOV, O. I.; NIKITINA, V. E. Intracellular lectins of *Lentinus edodes* at various developmental stages of the fungus. **Microbiology**, v. 77, n. 4, p. 441-445, 2008.

VETCHINKINA, E. P. et al. Activity and expression of laccase, tyrosinase, glucanase, and chitinase genes during morphogenesis of *Lentinus edodes*. **Microbiology**, v. 84, n. 1, p. 49-58, 2015.

VETCHINKINA, E. et al. Brown mycelial mat as an essential morphological structure of the shiitake medicinal mushroom *Lentinus edodes* (Agaricomycetes). **International journal of medicinal mushrooms**, v. 19, n. 9, 2017.

WICHERS, H. J. et al. Cloning, expression, and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. **Applied Microbiology and Biotechnology**, Oxford, v. 61, n. 4, p. 336-341, 2003.

YE, J. J. et al. Effects of active modified atmosphere packaging on postharvest quality of shiitake mushrooms (*Lentinula edodes*) stored at cold storage. **Journal of Integrative Agriculture**, v. 11, n. 3, p. 474-482, 2012.

ZHANXI, L.; ZHANHUA, L. Jun-cao technology. **China Agricultural Scientech press, Beijing**, p. 252, 2001.

ZIED, D. C. et al. Selection of strains for shiitake production in axenic substrate. **World Journal of Microbiology and Biotechnology**, v. 32, n. 10, p. 168, 2016.

ARTIGO 2

Physiological and ultrastructural characterization of the *in vitro* infection process of *Trichoderma* sp. in *Lentinula edodes*

Artigo será submetido de acordo com as normas da revista Brazilian Journal of Microbiology (Impact factor: 1.810)

Abstract

Lentinula edodes, one of the most important edible mushrooms in the world, is affected heavily by the infection of green mold, which is caused by *Trichoderma* sp. We collected the pathogen from diseased substrates to chacacterize and studied its effect on two *L. edodes* strains. The comparison between the two *L. edodes* strains showed that UFLA-LE5 presents a greater range of growth in environments considered atypical for its good development. However, the UFLA-LE6 strain produced brown barrage in the antithetic zone line of interaction with the pathogen, which has already been reported as a resistance strategy. On the other hand, the *Trichoderma* isolate showed a high tolerance to the different environmental conditions, besides a fast growth on *L. edodes*, proven fact with the scanning electron microscopy. We also visualized the pathogen coiling around the shiitake hyphae, which may be an attack feature of our isolate.

Keywords: Shiitake, green mold, Scanning electron microscopy.

Introduction

Lentinula edodes (Berk) Pegler is the most important edible mushroom in worldwide production (ROYSE; BAARS; TAN, 2017). In Brazil is widely cultivated, especially in the south and southest regions where the environmental conditions are more favorable (ISHIKAWA; KASUYA; VANETTI, 2001). The production of shiitake occurs in two ways: in bed-logs, a traditional method used over 900 years or in axenic cultivation in which sawdust blocks are supplemented with nutrients (ZHANXI; ZHANHUA, 2001). The incorporation of Shiitake culture into sawdust is a widely utilized technique that can assist in reducing the cost and time consumption associated with oak cultivation (KOO et al., 2018).

However, a wide range of pests and disease occurs in cultivation (EIRA, 2003). Mushroom yields and quality may be reduced by many diseases caused, for instance by *Diatrype* sp., *Hypocrea* sp. and *Nitschkia* sp. (BAK; KWON, 2005). Among them, fungal species in the genus *Trichoderma,* which commonly existed in soil, are the most important. *Trichoderma* is a fungal genus widely studied and used in the biological control of a range of phytopathogens (MACHADO; SILVA, 2013). They produce a large

range of secondary metabolites which have inhibitory activities, direct mycoparasitism as well as lysis and degradation of cell wall by enzymes (HOWELL, 2003).

From the perspective of *L. edodes*, green mold induced by *Trichoderma* sp. often attack and kill shiitake mycelia in bed-logs and reduce the mushroom yield (SEABY, 1998). The main species affecting *L. edodes* are *T. harzianum*, *T. viride*, *T. longibrachiatum*, and *T. polysporum* (JIANG; WANG, TAN, 1995). Shiitake cultures produce at least five straight-chain alcohols, which could act as antifungal substances (ISHIKAWA; KASUYA; VANETTI, 2001) and may play a role in the resistance to *Trichoderma* sp. (SAVOIE; MATA, 2003). It was also verified that under adequate nutritional conditions and favorable temperatures, some strains of *L. edodes* showed resistance to *Trichoderma* sp. (ISHIKAWA et al., 1980; BADHAM, 1991).

On the other hand, some studies explored environmental factors and cultivation conditions and their effect on mycelial growth of *L. edodes* and *Trichoderma* sp. (TOKIMOTO; KOMATSU 1995; BADHAM, 1991). Simultaneously, Bruce, Austin e King (1984) documented that a volatile of *Trichoderma* sp. could inhibit *L. edodes* mycelial growth. Nevertheless, the studies of the effect of *Trichoderma* species on *L. edodes* are less well-documented.

Several methods of controlling this pathogen have already been carried out, such as an accurate management control (PRZYBYLOWICK; DONOGHUE, 1990); spraying commercial alcohol or sodium hypochlorite solution (PASCHOLATI; STANGARLIN; PICCININ, 1998); logs immersion in calcium hydroxide solution (EIRA; MINHONI, 1996; ANDRADE, 1999); and fungicides (GIL, 1994; MATA; GAITÀN-HERNÁNDEZ, 1994). However, no method was fully effective in controlling the disease. One of the main reasons is that the genus *Trichoderma* presents a considerable genetic variability. Therefore, it is possible to find isolates with very different morphological and physiological characteristics (KLEIN; EVERLEIGH, 1998), even responsible for diversity in the antagonism to the same isolate (ETHUR et al., 2005; AULER; CARVALHO; MELLO, 2013; MACHADO; SILVA, 2013). Thus, the aim of the present work was to evaluate the mycelial growth of strains of *L. edodes* and *Trichoderma* sp. under different pH and temperatures conditions. Additionally, we analyze the *in vitro* antagonism of these fungal species via scanning electron microscopy.

Materials and Methods

Test organisms

The selected *Lentinula edodes* strains (UFLA-LE5 and UFLA-LE6) are deposited in the culture collection of the Edible Mushroom Laboratory at the Federal University of Lavras (UFLA), Lavras, Brazil. *Trichoderma* sp. used in this experiment was isolated from shiitake cultivation, and deposited in

the same collection. All of the fungal cultures were maintained on complete yeast medium (CYM; 2% glucose, 0.2% yeast extract, 0.2% peptone, 0.046% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1.8% agar) at 25ºC under a photoperiod of 12:12h light/dark cycles (WANG et al., 2016).

Light microscopy

The isolate of *Trichoderma* were incubated on CYM medium at 25°C, during which colony shape and pigment were documented. Four cover glasses were inserted slantingly into the CYM medium in order to observe the conidia and conidiophores via the microscope DM 2000, Leica (PARK; BAE; YU, 2006).

Effect of different temperature and pH treatments on mycelial growth of *L. edodes* and *Trichoderma* sp.

At the center of each plate a 5 mm diameter plug of testing species was placed with mycelium of the culture grown prior for 7 days in CYM. For the evaluation of different temperature treatments the plates were incubated at 15°C, 20°C, 25°C, 30°C, and 35°C and the medium at pH 6 for all conditions. Meanwhile for the assay of pH the values were 4, 5, 6, 7, 8, 9 and 10, adjusting the medium adding HC*l* or NaOH solution and all the plates were incubated at 25ºC. Estimations were conducted through daily measurements of the colony diameters until it reaches the dish edge, after which, the growth rate (GR) was determined, applying the equation adopted by Wang et al. (2016).

$$\text{Growth rate} = \frac{(\text{colony diameter} - \text{plug diameter})}{2\text{n}}$$

*where *n* represents cultivation days.

Antagonism interactions between *L. edodes* and *Trichoderma* sp. on *in vitro* system

Mycelial agar plugs (5 mm diameter) were cut from the growing front of 7-day-old colonies of *L. edodes* strains and inoculated on CYM at 1 cm from the edge. Seven days later, mycelial plugs of *Trichoderma* sp. cultures were inoculated in the same way but on the opposite side of the Petri dish. All the dishes were incubated at 25ºC. The shiitake inoculum were prepared 7 days before *Trichoderma* inoculation because of its stabilization before *Trichoderma*'s attack. The interacting zones between *L.*

edodes and *Trichoderma* sp. were cut at 48, 72 and 96 hours post inoculation and analyzed by scanning electron microscopy (SEM) (INBAR; MENENDEZ; CHET, 1996).

Effect of *Trichoderma* fermentation broth on *L. edodes* mycelium

In this assay *Trichoderma* sp. plugs (5 mm diameter) were inoculated into the PD (potato dextrose) broth, followed by 1 week culture in darkness at 25°C, 160 rpm. Mycelium was removed by filtration, and the filtrate was added to the CYM medium to thirty percent in volume, with 30% sterile water used for control. *L. edodes* mycelium plugs (5 mm diameter) were inoculated on the medium and cultured in darkness at 25°C. Sample collection was done 10 days later and the changes of *L. edodes* mycelium were observed via SEM.

Scanning electron microscopy

Fixing of these samples was then done in a modified Karnovsky's solution (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.05M sodium cacodylate buffer, 0.001M CaCl2, pH 7.2) and treated according to the method of ALVES et al. (2013). Images produced were recorded and studied using the Photopaint software of the Corel Draw X6 package.

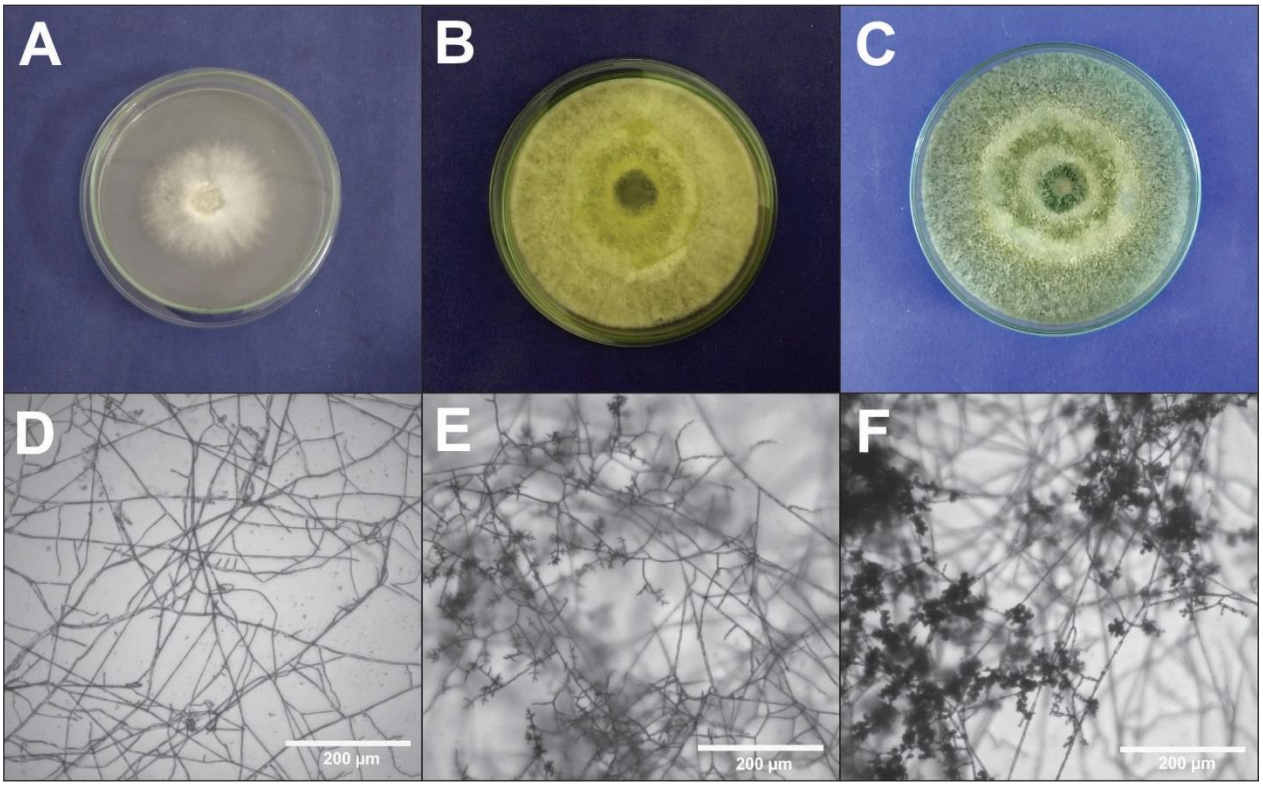
Results and Discussion

Morphology of *Trichoderma* sp.

The characteristics of *Trichoderma* isolate could seen in the figure 1. At first day the growth mycelial it is beggining and the color of the colony is white; subsequently, the color becomes light green and the dish is already colonized at second day; finally, at third day the color changed to dark green because of conidia clusters generation. This behavior pattern has already been reported in other studies (GAMS; BISSETT, 1998; PARK; BAE; YU, 2006; WANG et al., 2016).

Besides colony morphology the conservative regions sequencing is a powerful tool for identification of *Trichoderma* species (WANG et al., 2016). From the perspective of diseased shiitake logs some *Trichoderma* species have already related, such as *T. harzianum, T. atroviride, T. viride, T. pleuroticola, T. longibrachiatum* and *T. oblongisporum* (TOKIMOTO; KOMATSU, 1995; JIANG; WANG; TAN, 1995; SAVOIE; MATA; BILLETTE, 1998; LEE et al., 2008; PARK; BAE; YU, 2006).

Figure 1 - Colony and microscopic characteristics of *Trichoderma* sp. isolate in different times, respectively. (A and E) 24 hours, (B and E) 48 hours and (C and F) 72 hours after inoculation in CYM medium.



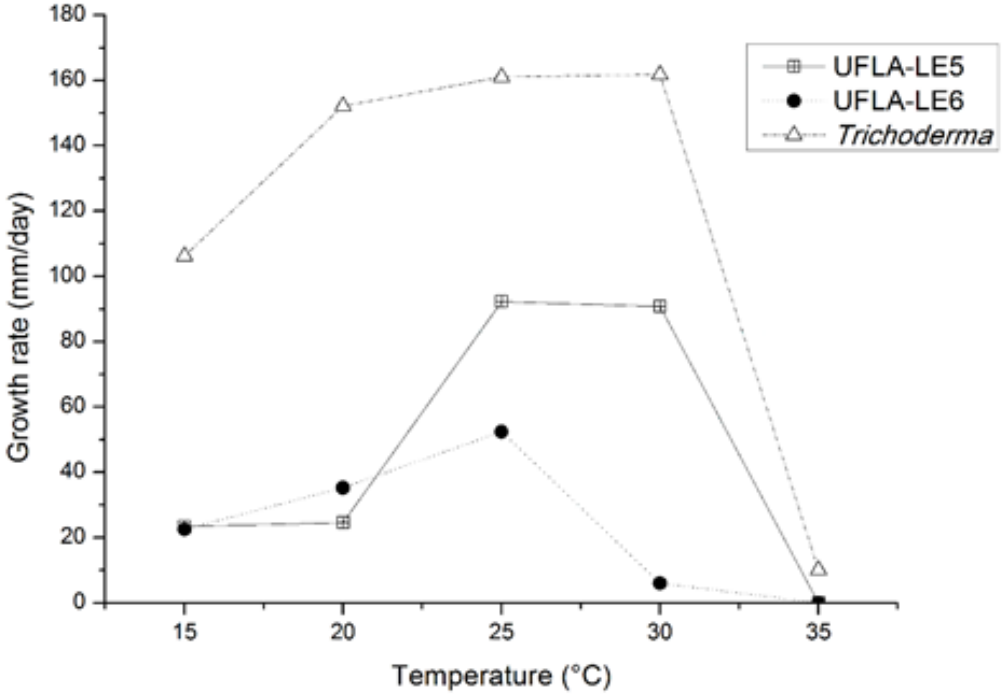
Effect of different temperatures and pH on *L. edodes* and *Trichoderma* sp. mycelia

According to figure 2 all the isolates started with a relatively low growth rate, subsequently reached a peak and, finally, had their growth reduced as the temperature increased. The highest growth rate for *Trichoderma* was 161.78 mm/d at 30ºC, while for UFLA-LE5 and UFLA-LE6 strains it was at 25ºC with 92.24 mm/d and 52.42 mm/d, respectively. According Wang et al. (2016), the optimum growth temperature for *Trichoderma* strains (T39 and T21) was also 30ºC while in other study *Trichoderma* isolates presented the highest growth between 22-25ºC (SINGH et al., 2014). Which explains the fact of the optimal range for mycelial growth of the genus *Trichoderma* can vary between 20-30ºC (BOMFIM et al., 2010). From the perspective of *L. edodes* mycelia, the strains Yuhua-2 and Xiang939 grew likewise well at 25°C, and the average growth rate of mycelia were 5.57 mm/d and 4.16 mm/d, respectively (WANG et al., 2016). In the same way, *L. edodes* radial growth was highest when incubation temperature was greater than 25ºC and decreased at temperatures above 30ºC and below 20ºC (KHAN et al., 1991).

Another interesting fact is that *Trichoderma* isolate could grow at all temperatures, despite having the lowest growth rate at 35ºC (10.13 mm/d), while neither *L. edodes* strains grew at 35ºC. These data

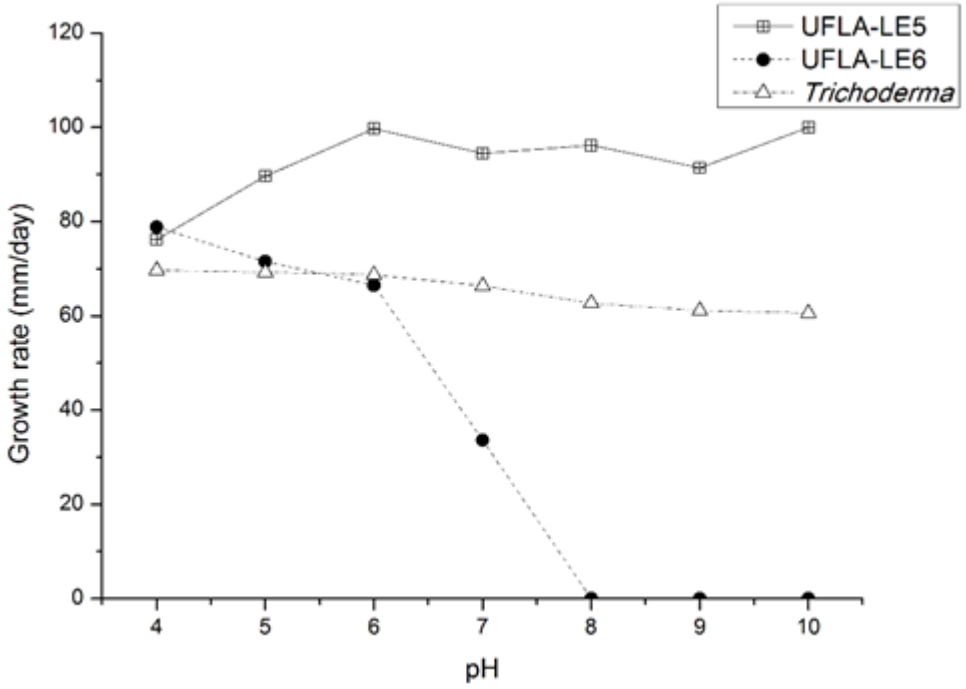
were similar as those reported by WANG et al. (2016), including the lowest growth rate of *Trichoderma* (7.05 mm/d), however Jaill et al. (2006) have affirm that *Trichoderma* isolates normally do not grow at temperatures below 17°C.

Figure 2 - Effect of different temperature treatments on mycelia growth of two *L. edodes* (UFLA-LE5 and UFLA-LE6) strains and one *Trichoderma* sp. isolate.



Analyzing the pH effect on mycelial growth, we observe that the *Trichoderma* and the UFLA-LE5 strain could grow normally at a pH range 4-10 (Figure 3). These results corroborate with those showed by Wang et al. (2016) and Hhiroko Hassegawa, Megumi Kasuyaa and Dantas Vanetti (2005). However, the UFLA-LE6 strain exhibit a decrease on growth rate at pH 7 (33.61 mm/d) and from pH 8 it was no longer able to grow. The suitable pH range for growth mycelial of all isolates was between 4-6. The pH about 4.5 is considered by some authors as the optimum value for biomass production of *L. edodes* (SONG; CHO; NAIR, 1987; KOMEMUSHI; YAMAMOTO; FUJITA, 1995). In addition, the growth rate of *Trichoderma* isolate was significantly faster than that *L. edodes* strains in the same condition.

Figure 3 - Effect of different pH treatments on mycelia growth of two *L. edodes* (UFLA-LE5 and UFLA-LE6) strains and one *Trichoderma* sp. isolate.

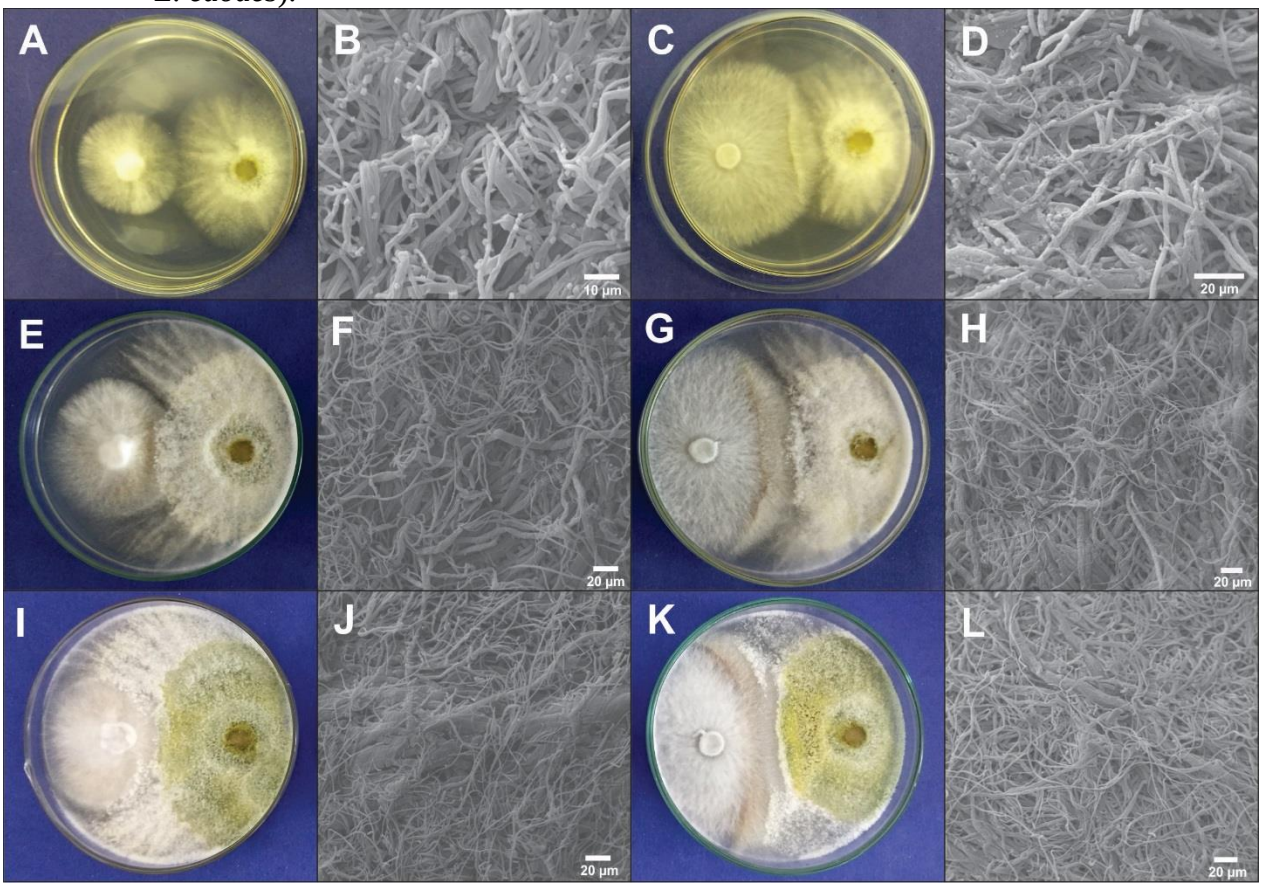


Several environmental factors, for instance temperature, humidity and sunshine, affect sharply the activity of species varied under different conditions, which may influence their distribution (CAO; BIAN; XU, 2014). In this context, it was found that environmental conditions where different *Trichoderma* species live had obvious region distribution and changed as the season, which had an important role in *Trichoderma* sp. growth (WIDDEN; SCATTOLIN, 1988).

Effect of *Trichoderma sp.* on *L. edodes* mycelia

At first hours of incubation, the strain UFLA-LE5 were slightly invaded by *Trichoderma* sp. while in UFLA-LE6 was possible to observe the formation of a deadlock (Figure 4A, C). After three days, *L. edodes* strains were partially invaded by *Trichoderma* isolate (Figure 4E, G). At 96 hours of incubation, UFLA-LE6 strain stopped growing with the formation of strong line of antithetic zone with *Trichoderma*, whereas the UFLA-LE5 strain was highly invaded by the pathogen (Figure 4I, K).

Figure 4 - Colony photographs and scanning electron micrographs of features of antagonism between *Trichoderma* sp. and *L. edodes* strains over time (right: *Trichoderma*, left: *L. edodes*).



(A, E and I) 48, 72 and 96 hours after inoculation of UFLA-LE5 strain in CYM medium, (B, F and J) corresponding micrographs of UFLA-LE5, (C, G and K) 48, 72 and 96 hours after inoculation of UFLA-LE6 strain in CYM medium, (D, H and L) corresponding micrographs of UFLA-LE6.

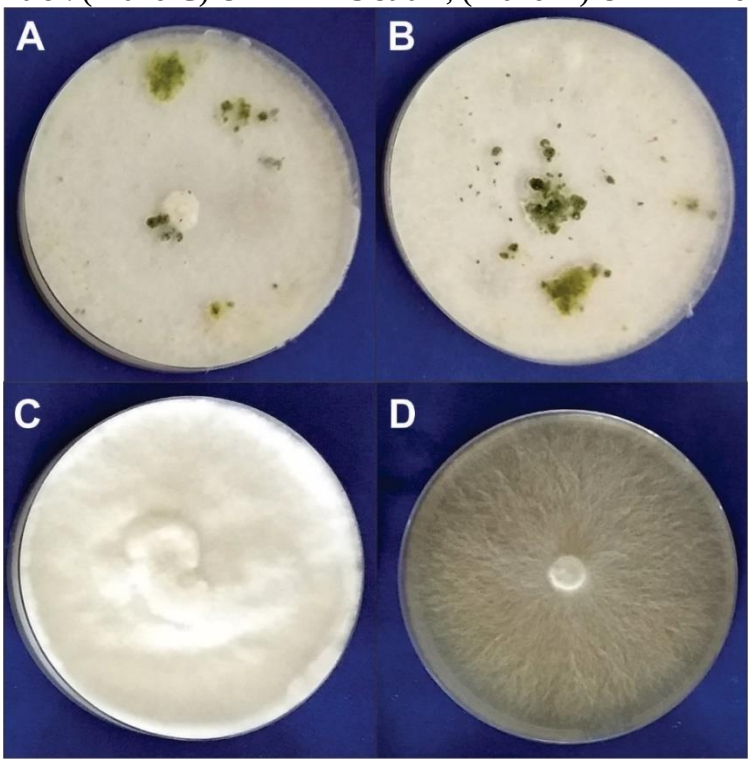
In dual culture, Lee et al. (2008) working with 11 and 5 strains of *L. edodes* and *Trichoderma* sp., respectively, observed the same behavior during one month. On the other hand, Reper and Penninckx (1987) observed that *Trichoderma* affected the growth of *Pleurotus ostreatus* mainly by producing a volatile toxin. Interestingly, we may observe that the UFLA-LE6 strain is more susceptible to physical conditions changes, however, presents a higher resistance to the pathogen.

In our study, UFLA-LE6 strain mycelium formed a brown barrage in the antithetic zone line with *Trichoderma* sp., which could be associated with the formation of melanin compounds. Some studies suggest that melanin formation is part of a defensive response against mycelial invasion and that these compounds help fungi to adapt to environmental stress (RAYNER; GRIFFITH; WILDMAN, 1994; BADALYAN; INNOCENTI; GARIBYAN, 2004).

From the perspective of *Trichoderma* sp. fermentation broth effect, the mycelial growth of *L. edodes* strains was slowly than the control; which may indicates the influence of some metabolites

produced by this pathogen in the mycelial growth of shiitake strains. After 10 days *Trichoderma* mycelium grew forming irregular conidial clusters, which corroborates the result of Wang et al. (2016) (Figure 5).

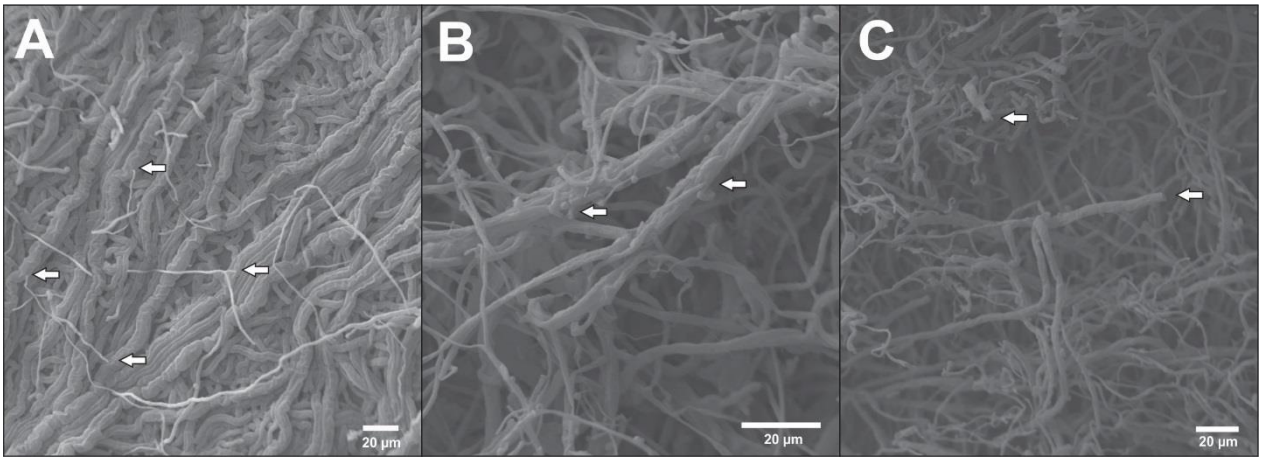
Figure 5 – Photographs of 10-day-old colonies of *L. edodes* strains in CYM medium added of 30% of *Trichoderma* broth fermentation and their control. (A and C) UFLA-LE5 strain, (B and D) UFLA-LE6 strain.



Interaction between *L. edodes* and *Trichoderma* sp. in SEM

Using the scanning electron microscope, an increase in the *Trichoderma* sp. prevalence was noticed over time, demonstrating the infection occurrence (Figure 4). Evidences of direct penetration of the pathogen into shiitake were also observed (Figure 6A). *Trichoderma* sp. hyphae could coil around the hyphae of *L. edodes* (Figure 6B) and after some time the *L. edodes* mycelia became ruptured and rough (Figure 6C), which are consistent with the results of the interaction between *L. edodes* and *Trichoderma* sp. (WANG et al., 2016).

Figure 6 - Scanning electron micrographs of *L. edodes* inoculated with *Trichoderma* sp. (A) pathogen hyphae penetration on shiitake, (B) mycoparasitism, (C) ruptured hyphae of *L. edodes*.



L. edodes hyphae could get broken by two reasons, by coiling (mycoparasitism), or produce a wealth of enzymes, such as, chitinase, β-glucanases, anthraquinones, and isocyano metabolites (SAVOIE; MATA; BILLETTE, 1998; SAVOIE; MATA, 2003). Several studies showed that *Agaricus bisporus* had the mycelial walls attacked by hydrolytic enzymes produced by different *Trichoderma* sp (WILLIAMS et al., 2003; GUTHRIE; KHALIF; CASTLE, 2005; GUTHRIE; CASTLE, 2006).

It would be very interesting to investigate the *Trichoderma* isolate species and to test these shiitake strains with other *Trichoderma* species, in order to obtain a behavior profile of these strains against the pathogen. Therefore, the understanding of these particular interactions may be a useful key to the control of greenmold disease during shiitake cultivation.

Acknowledgements

The FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) are acknowledged for financial support.

References

ALVES, E. et al. Scanning electron microscopy for fungal sample examination. In: **Laboratory Protocols in Fungal Biology**. Springer, New York, NY, p. 133-150, 2013.

ANDRADE, F. A. Efeitos de fungos contaminantes na produção de Shiitake (*Lentinula edodes* (Berk.) Pegler) em toros de *Eucalyptus saligna* SM. **Dissertação (Mestrado em Agronomia)** - Universidade Estadual Paulista, Faculdade de Ciências Agronômicas, Botucatu, 1999.

AULER, A. C. V.; CARVALHO, D. D. C.; MELLO, S. C. M. Antagonismo de *Trichoderma harzianum* a *Sclerotium rolfsii* nas culturas do feijoeiro e soja**. Revista Agro@mbiente**, v. 7, p. 359-365, 2013. Disponível em: revista.ufrr.br/index.php/agroambiente.

BADHAM, E. R. Growth and competition between *Lentinus edodes* and *Trichoderma harzianum* on sawdust substrates. **Mycologia**, Lawrence, v. 83, n. 4, p. 455-463, 1991.

BADALYAN, S. M.; INNOCENTI, G.; GARIBYAN, N. G. Interactions between xylotrophic mushrooms and mycoparasitic fungi in dual-culture experiments. **Phytopathologia Mediterranea**, v. 43, n. 1, p. 44-48, 2004.

BAK, W. C.; KWON, H. J. Biology and control of pests and diseases in shiitake log cultivation. **Mushroom Grower's Handbook**, 2 ed., p. 152-161, 2005.

BOMFIM, M. P. et al. Antagonic effect *in vitro* and *in vivo* of *Trichoderma* sp. to *Rhizopus stolonifer* in yellow passion fruit. **Summa Phytopathologica**, v. 36, n. 1, p. 61-67, 2010.

BRUCE, A.; AUSTIN, W. J.; KING, B. Control of growth of *Lentinus edodes* by volatiles from *Trichoderma*. **Transactions of the British Mycological Society**, v. 82, n. 3, p. 423-428, 1984.

CAO, X. T.; BIAN, Y. B.; XU, Z. Y. First report of *Trichoderma* oblongisporum causing green mold disease on *Lentinula edodes* (shiitake) in China. **Plant Disease**, v. 98, n. 10, p. 1440-1440, 2014.

EIRA, A. F**.** Cultivo do cogumelo medicinal *Agaricus blazei* (Murrill) ss. Heinemann. **Viçosa: Aprenda Fácil**, 2003.

EIRA, A. F.; MINHONI, M. T. A. Cultivo de cogumelos comestíveis. **Botucatu: ELO/Instituto de Economia Associativa**, 1996.

ETHUR, L. Z. et al. Fungos antagonistas a *Sclerotinia sclerotiorum* em pepineiro cultivado em estufa. **Fitopatologia Brasileira**. v. 30, p. 127-133, 2005.

GAMS, W.; BISSETT, J. Morphology and identification of *Trichoderma*. In: GAMS, W.; BISSETT, J.; MALIK, A. F. ***Trichoderma* and *Gliocladium***, v. 1, p. 3–34, 1998.

GIL, F. H. Introducción al cultivo de Shiitake (*Lentinus edodes*). In: **I Jornadas técnicas del champiñón y otros hongos comestibles en Castilla-La Mancha: celebradas en Motilla del Palancar (Cuenca), 3 y 4 de noviembre de 1993**. Diputación Provincial de Cuenca. Patronato de Promoción Económica, p. 143-150, 1994.

GUTHRIE, J. L.; KHALIF, S.; CASTLE, A. J. An improved method for detection and quantification of chitinase activities. **Canadian journal of microbiology**, v. 51, n. 6, p. 491-495, 2005.

GUTHRIE, J. L.; CASTLE, A. J. Chitinase production during interaction of *Trichoderma* aggressivum and *Agaricus bisporus*. **Canadian journal of microbiology**, v. 52, n. 10, p. 961-967, 2006.

HIROKO HASSEGAWA, R.; MEGUMI KASUYA, M. C.; DANTAS VANETTI, M. C. Growth and antibacterial activity of *Lentinula edodes* in liquid media supplemented with agricultural wastes. **Electronic Journal of Biotechnology**, v. 8, n. 2, p. 94-99, 2005.

HOWELL, C. R. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. **Plant disease**, v. 87, n. 1, p. 4-10, 2003.

INBAR, J.; MENENDEZ, A. N. A.; CHET, I. Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. **Soil Biology and Biochemistry**, v. 28, n. 6, p. 757-763, 1996.

ISHIKAWA, H. et al. Physiological changes in *Lentinus edodes* (Berk.) Sing. mycelia induced by *Trichoderma* metabolites. **Reports of the Tottori Mycological Institute**, n. 18, p. 197-204, 1980.

ISHIKAWA, N. K.; KASUYA, M. C. M.; VANETTI, M. C. D. Antibacterial activity of *Lentinula edodes* grown in liquid medium. **Brazilian Journal of Microbiology**, São Paulo, v. 32, n. 3, p. 206-210, 2001.

JAILL, C. et al. **Efecto de la temperatura sobre el crecimiento micelial de *Trichoderma harzinum* T-39 y la relación interbiótica com *Botrytis cinera* procedente de tomate**. Dissertação (Mestrado) - Pontificia Universidad Católica de Chile, Faculdad de Agronomia, Santiago, 2006.

JIANG, H. F.; WANG, F. L.; TAN, Q. A preliminar study on *Trichoderma* sp. and dominant *T.* species in *Lentinula edodes* growing. **Acta Agriculturae Shanghai,** v. 11, p. 85–90, 1995.

KHAN, S. M. et al. Studies on shiitake mushroom (*Lentinula edodes* (Berk.) Pegler). **Science and Cultivation of Edible Fungi**, v. 2, p. 503-507, 1991.

KLEIN, D.; EVERLEIGH, D. E. Ecology of Trichoderma. In: KUBICEK, C.; PENTTILÄ, M. **Trichoderma and Gliocladium: Enzymes, biological control and commercial applications**. CRC Press, p. 49-72, 1998.

KOMEMUSHI, S.; YAMAMOTO, Y.; FUJITA, T. Antimicrobial substance produced by *Lentinus edodes*. **Journal of Antibacterial and Antifungal Agents Japan**, v. 23, n. 2, p. 81-86, 1995.

KOO, J. et al. Browning technology for shiitake in sawdust using LED source. **한국버섯학회지**, v. 16, n. 4, p. 331-333, 2018.

LEE, H. M. et al. Breeding and screening of *Lentinula edodes* strains resistant to *Trichoderma* sp. **Mycobiology**, v. 36, n. 4, p. 270-273, 2008.

MACHADO, D. F. M.; SILVA, A. C. F. *Trichoderma* no controle *in vitro* de fungos presentes em diásporos de *Gochnatia polymorpha*. **Revista de Ciências Agrárias**, v. 36, p. 07-09, 2013.

MATA, G.; GAITÀN-HERNÁNDEZ, R. Avances en el cultivo de shiitake en pulpa de café. **Revista Iberoamericana de Micologia**, Bilbao, v. 11, n. 1, p. 90-91, 1994.

PARK, M. S.; BAE, K. S.; YU, S. H. Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. **Mycobiology**, v. 34, n. 3, p. 111-113, 2006.

PASCHOLATI, S. F.; STANGARLIN, J. R.; PICCININ, E. **Cogumelos**: cultivo e comercialização (shiitake e cogumelo do sol). Cuiabá: Sebrae/MT, (Coleção agroindústria, 17), p. 85, 1998.

PRZYBYLOWICZ, P.; DONOGHUE, J. **Shiitake grower's handbook**: the art and science of mushroom cultivation. Dubuque: Kendall, p. 217, 1990.

RAYNER, A. D. M.; GRIFFITH, G. S.; WILDMAN, H. G. Induction of metabolic and morphogenetic changes during mycelial interactions among species of higher fungi. **Biochemical Society Transactions**, v. 22, p. 389-394, 1994.

REPER, C.; PENNINCKX, M. Inhibition of *Pleurotus ostreatus* growth and fructification by a diffusible toxin from *Trichoderma hamatum*. **Journal of Food Science and Technology**, v. 20, p. 291-292, 1987.

ROYSE, D. J.; BAARS, J.; TAN, Q. Current overview of mushroom production in the world. **Edible and medicinal mushrooms:** technology and applications, p. 5-13, 2017.

SAVOIE, J. M.; MATA, G.; BILLETTE, C. Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. **Applied Microbiology and Biotechnology**, v. 49, n. 5, p. 589-593, 1998.

SAVOIE, J. M.; MATA, G. *Trichoderma harzianum* metabolites pre-adapt mushrooms to *Trichoderma aggressivum* antagonism. **Mycologia**, v. 95, n. 2, p. 191-199, 2003.

SEABY, D. *Trichoderma* as a weed mould or pathogen in mushroom cultivation. **Trichoderma and Gliocladium**, v. 2, p 267-272, 1998.

SINGH, A. et al. Optimal physical parameters for growth of *Trichoderma* species at varying pH, temperature and agitation. **Virology & Mycology**, v. 3, n. 1, p. 1-7, 2014.

SONG, C. H.; CHO, K. Y.; NAIR, N. G. A synthetic medium for the production of submerged cultures of *Lentinus edodes*. **Mycologia**, v. 79, n. 6, p. 866-876, 1987.

TOKIMOTO, K.; KOMATSU, M. Selection and breeding of shiitake strains resistant to *Trichoderma* sp. **Canadian Journal of Botany**, v. 73, n. S1, p. 962-966, 1995.

WANG, G. et al. Diversity and effect of *Trichoderma* sp. associated with green mold disease on *Lentinula edodes* in China. **Microbiology Open**, v. 5, n. 4, p. 709-718, 2016.

WIDDEN, P.; SCATTOLIN, V. Competitive interactions and ecological strategies of *Trichoderma* species colonizing spruce litter. **Mycologia**, v. 80, p. 795-803, 1988.

WILLIAMS, J. et al. Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. **Applied and environmental microbiology**, v. 69, n. 7, p. 4192-4199, 2003.

ZHANXI, L.; ZHANHUA, L. Jun-cao technology. **China Agricultural Scientech press, Beijing**, p. 252, 2001.

ARTIGO 3

Transcriptome analysis of *Lentinula edodes* strains and description of genes involved in substrate colonization and the brown film formation by using RT-qPCR

Artigo será submetido de acordo com as normas da revista Applied and Environmental Microbiology (impact factor: 4.310)

Abstract

The success of shiitake cultivation requires a strategic process, the brown film formation, which promotes a high yield of mushrooms when well formed. Thus, the comparison between a productive and a less productive strain may be an interesting approach to studying the genetic processes involved in this process. Transcriptomic analyzes are powerful tools to provide an understanding of gene regulation and followed by RT-qPCR is probably the best option to analyze the expression patterns of certain genes under different conditions. Therefore, we aimed to analyze the level of gene expression for over-represented transcripts as well as the differences between the expression of *L. edodes* genes under different culture conditions and stages of growth. Were utilized two strains of *L. edodes*, UFLA-LE5 (less-productive) and UFLA-LE6 (productive), and three sampling during the cultivation cycle. In addition, we tested the suitability of 10 reference genes and analyzed the relative expression of 14 highly expressed genes in the transcriptome using RT-qPCR. Our results showed that the UFLA-LE5 strain has more upregulated genes than the UFLA-LE6 strain, which seems contradictory; however, it may suggest a failure of the strain UFLA-LE5 to control the level of the genetic expression in order to guarantee only the most important genes to be highly expressed during the browning process. The alcohol dehydrogenase, conservative protein, hydrolase and transferase genes always exhibited a decrease in expression on the outside sample of UFLA-LE6 strain at 90 days. This profile can be explained by the speed and stability with which the brown film is formed in this strain.

Keywords: Brown mycelial layer. RNA-seq. Quantitative PCR.

Introduction

Lentinula edodes (Berkley) Pegler, known as shiitake, has been cultivated traditionally in natural logs of oak, however, this cultivation is time consuming and energy intensive and difficult to handle on a large scale (DIAS, 2010). For this reason, as has been happening worldwide, the shiitake growers have adopted the axenic cultivation in sawdust-based synthetic logs (DIAS, 2010). In which, shiitake stands out from other mushroom species, by requesting the formation of a brown film on the surface of the substrate

before the primordium initiation (ROYSE, 2009). Therefore, the brown film formation is a strategic step to have a high mushroom yield and needs for a special attention during the cultivation cycle. In a previous study (ZIED et al., 2016) was observed the differences of productivity in different strains of shiitake cultivated in Brazil. Besides, it was found out a strain that did not produce the brown film and, consequently, did not produce any mushroom. The comparison between a productive and a less productive strain may be a very interesting approach to study the genetic and biochemical processes involved in the brown film formation.

In the last years, a couple of works has been published about transcriptomic analyses to investigate the candidate genes involved in the brown film formation. Generally, these studies use the approach of analysis in function of the time of incubation and light exposition (TANG et al., 2013). This approach has allowed some insights about the process, however, the brown film formation is too complex and many questions still remain. It is known that transcriptomic analyses are powerful tools for providing an understanding of gene regulation and the presumptive function of genes; and reverse transcription (RT) followed by real-time PCR (RT-quantitative PCR [RT-qPCR]) is likely the best option for analyzing the expression patterns of certain genes under multiple conditions.

RT-qPCR it is the most reliable technique for validating RNA-seq data because of its specificity, reproducibility, and capacity for detecting and measuring tiny amounts of nucleic acids in a wide quantification range (PFAFFL, 2004). Since its first appearance in 1993 (HIGUCHI et al., 1993), many studies have been performed to improve the accuracy of RT-qPCR (PFAFFL, 2001; TICHOPAD et al., 2003; SPIESS; FEIG; RITZ, 2008; RUIJTER et al., 2009). Besides, several types of software that implement these approaches are currently available (PFAFFL; HORGAN; DEMPFLE, 2002; HELLEMANS et al., 2007). Within the two possible quantification strategies (relative and absolute), relative quantification is a very common strategy for analyzing the expression levels of a target gene under multiple conditions or in multiple samples (CASTANERA et al., 2015).

Therefore, in this work we showed the level of gene expression for some transcripts considered over-represented as well as the differences between the expression of genes of *L. edodes* in different growing stages. We tested the suitability of 10 candidate genes for their use as reference genes in studies with *L. edodes* and analyzed the relative expression of 14 highly expressed genes in the transcriptome by using RT-qPCR.

Material and Methods

Mycelium production and substrate cultivation

As a control of the experiment, the strains UFLA-LE5 and UFLA-LE6 were cultivated in Potato Dextrose Broth (Oxoid) at 25º C for 25 days, under static conditions. *Eucaliptus* sawdust-based substrate was utilized to cultivate the fungal strains as described by Zied et al. (2016). After inoculation, the bags were incubated at room temperature (25 ± 2ºC) during three months.

Sample collection and nucleic acid extraction

In order to analyze the brown film formation in different times, the samples were collected at 30, 60 and 90 days after inoculation of the bags. At each collection time, samples were taken from the surface and from inside the blocks. Besides, three blocks were utilized from each strain. The mycelium grown in liquid medium was filtered after 25 days, for the two strains. All the samples were grounded in a sterile mortar with liquid nitrogen, distributed in microtubes and stored at -80ºC. Total RNA was extracted from 200 mg of deep-frozen tissue using a fungal RNA E.Z.N.A. kit (Omega Bio-Tek, Norcross, GA, USA) and its integrity was estimated by denaturing electrophoresis on 1% (w/v) agarose gels. Nucleic acid concentrations were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the purity of the total RNA was estimated by the 260 nm/280 nm absorbance ratio. The DNase treatment was performed in the middle of the extraction, following the guidelines of the manufacturer of RNase-Free DNase I Set kit (Omega Bio-Tek, Norcross, GA, USA).

Sequencing and data analysis

The samples were sent for sequencing and after transcriptome bioinformatics analysis we calculate average, median and standard deviation for data normalization. The highly expressed genes per library were determined using FPKM average plus 3-fold the standard deviation (AVG + 3STD). Subsequently, we found these genes among libraries from primary data and looked for annotation of the differentially expressed genes.

Primer design

From the FPKM data, we selected 10 genes with the lowest coefficient of variation as reference genes candidates (Table 1). Genes were chosen on the basis of stable expression in our RNA-seq experiment. The 10 candidates were classified according to their suitability as internal standards using the geNorm (VANDESOMPELE et al., 2002) and NormFinder (ANDERSEN; JENSEN; ØRNTOFT, 2004) algorithms, and the 3 with the best results were chosen.

In table 2 are the 13 genes of interest tested, which should had a high FPKM ratio between the outside and inside of the substrate. In this way, we selected genes that were very expressed in the brown cover and could be associated to their formation. Primers were designed using transcripts sequences and the Primer-Quest^{SM} tool (Integrated DNA Technologies, Madrid, Spain).

Table 1 - Transcript identification (gene, UFLA-LE5 and UFLA-LE6) and PCR primers used (type – forward or reverse – and sequences) for the reference genes selected.

Transcript identification			**Primers used**	
Gene	**UFLA LE5**	**UFLA LE6**	**Type***	**Sequence**
Protein Fcf2	TR5587	TR6772	Fw	GGCTTCATACTCCGCTTTCT
			Rv	GGAGGGTGCGAATGGTTATTA
Intracellular transport protein	TR1392	TR2747	Fw	CGGCTGCTGTTAGGGATTT
			Rv	CCCAGGTTCACGGTTGTATT
ATP synthase 1	TR1088	TR7597	Fw	CGGGATACACACGAACTCATT
			Rv	AGAGACAGTCGCGGAAGTA
RNA methylase	TR5002	TR5249	Fw	GAAGAGTTGGTCGCTGTTCT
			Rv	GCTGGGCAGAAGAAGGTATT
Kinase 1	TR2149	TR8280	Fw	GGCCGGGAGGATCATTTATT
			Rv	GGCTGACACCTTTGGGATATAG
ATP synthase 2	TR1088	TR7597	Fw	GCGTGCATCTTTGCCTAATG
			Rv	CCCACCACATAGCCTGATTAC
Kinase 2	TR387	TR8957	Fw	GTGCAGGATGGTGACGTTAT
			Rv	CCCATGCTTTCCTCCTCTTT
Integral membrane protein	TR463	TR7338	Fw	CTATGGGCATCGGAGGATTT
			Rv	CTTCGACCTCTCTCTCCATTTC
Protein GVP36	TR2680	TR169	Fw	CCAAGCGGAAGGTAAACAAATC
			Rv	GTCGCAGTCTCTCCAATCAA
Major facilitator superfamily	TR5032	TR5322	Fw	GTCCACTGCTGGAAGAACAT
			Rv	GGAGTTTGAGGGCAGAGATAAG

* Fw and Rv, forward and reverse primer types.

Table 2 - Transcript identification (gene, UFLA-LE5 and UFLA-LE6) and PCR primers used (type – forward or reverse – and sequences) for the interest genes selected.

Transcript identification			**Primers used**	
Gene	**UFLA LE5**	**UFLA LE6**	**Type***	**Sequence**
Aldehyde reductase	TR2781	TR5952	Fw	CTTCCATCGCTCTTGCTGTA
			Rv	GTGAGGGTGCCTATACTCATTT
Alcohol dehydrogenase 1	TR1286	TR5513	Fw	TTGCATCCTCGCTGTCTTT
			Rv	ACCATGCTTACTGTCGCTATT
D-Aspartate oxidase	TR579	TR533	Fw	GGGTCGAATGTCAGAGTTGAG
			Rv	CAGCACATCCAAACGACAAAG
Conserved protein	TR790	TR6025	Fw	TCCACTGCGACCTTCATAAC
			Rv	CGTGGTTCGTAGATTGCCTTA
Hydrolase/Transferase	TR8749	TR429	Fw	GTGATTCCAGGCTTCGTTACT
			Rv	GGAGAACAGGCGTCATCTATC
3-Oxoacyl CoA thiolase 1	TR142	TR2963	Fw	AGCAGAACGAATAGCTGAGAC
			Rv	CCACCACCACTACCTCTTTATC
Hydrolase	TR7339	TR107	Fw	CACCCGGAAGGGCAATAATA
			Rv	CCTCCGCAAATGGTGTAAGA
Transferase	TR5782	TR6969	Fw	CAGCGAACAGTCGGAGAATAG
			Rv	TACGCGTGTCAGGATCTTAATG
Alcohol dehydrogenase 2	TR1226	TR7601	Fw	GCTCAGTCAGGCCAAAGAA
			Rv	GGACCGCAACGAAATGTAATG
Thioredoxin reductase	TR2882	TR2948	Fw	GATGGTGCCGTTCCTATCTT
			Rv	GCGACGAACGAGAACATAAAC
3-Oxoacyl CoA thiolase 2	TR4275	TR1421	Fw	GCCCATCATTCCTCTTGGATAG
			Rv	TCGGTGAGCTTGTGGTATTG
Cytochrome C	TR1573	TR9170	Fw	CGCGTCTTTATTGCCACATTAC
			Rv	GGTCTACATCGGTCGTCTAGTA
Non functional annotation	TR2781	TR2915	Fw	CGATCTGTCACCGTTCTTCTT
			Rv	TGTCAACTGGGCGTGATAAG

* Fw and Rv, forward and reverse primer types.

Reverse transcription and real-time qPCR

Total RNA (800 ng per sample) was reverse transcribed into cDNA in a 20 µL volume using the iScript cDNA Synthesis kit (Bio-Rad, Alcobendas, Spain). Subsequently, the samples were purified using an OneStep™ PCR Inhibitor Removal kit (Zymo Research, California, USA). RT-qPCR experiments were performed using a CFX96 (Bio-Rad Laboratories, S.A.) thermal cycler. SYBR green fluorescent dye was used to detect product amplification. Each reaction mixture was set to a final volume of 20 µL containing 10 µL iQ SYBR green supermix from Bio-Rad, 2 µL of 300 µM forward and reverse primers, 1 µL of 1:10 dilution of cDNA and 5 µL nuclease-free water.

The amplification program consisted of 5 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 63°C, and 15 s at 72°C; followed by 1 min at 95ºC and 1 min at 63ºC; and a final melting curve analysis in which the temperature was increased at increments of 0.5°C every 5 s in a linear gradient from 65 to 95°C. The specificity of each reaction was confirmed by inspection of the melting curve profiles. Each reaction was performed in triplicate, and nontemplate controls (NTCs) were included for each primer set. An experimentally validated interplate calibrator (IPC) was used to compensate for interplate variation. Data processing was carried out using GenEx software (MultiD Analyses).

Results

Raw data and genome coverage

Presentation of the raw data in terms of number of reads, bases sequenced and genome coverage obtained for *L. edodes* transcriptomes run in Illumina machines is shown on table 3. The sequencing of samples in high-throughput provided a deep genome coverage (higher than 100x) in all libraries analyzed.

Table 3 - Raw data and genome coverage per library.

Library	Sample	Number of reads	Total number of bases*	Genome coverage
LE5_5E	Inside	54.377.414	5.437.741.400	118.21
LE5_6C	Surface	52.093.760	5.209.376.000	113.25
LE5_M	Mycelium	60.173.690	6.017.369.000	130.81
LE6_1A	Inside	52.932.554	5.293.255.400	115.07
LE6_2C	Surface	46.906.928	4.690.692.800	101.97
LE6_M	Mycelium (C)	53.524.918	5.352.491.800	116.36

* The total number of bases consist primarily from the number of reads multiplied by a factor of 100, once the size of the Illumina reads were 100bp.

Analyses of highly expressed genes per library

FPKM is a measure of gene expression and it was considered the most consistent among all libraries found. The average expression found for a gene for each million reads sequenced was ~67 fragments found per kilobase, with a standard deviation of ~155 and median value of ~27 fragments. The total number of pb genes is 9137. The FPKM information and number of genes per FPKM limit are showed in the tables 3 and 4.

Table 4 - General analysis of the transcriptomic differences between UFLA-LE5 and UFLA-LE6 strains.

Parameter	**UFLA-LE5**	**UFLA-LE6**
Total Nº of transcripts	13.203	12.895
Nº of transcripts with annotation	3.535 (26.77%)	4.066 (31.53%)
Nº of transcripts with FPKM higher than 1.000	7	7
Nº of transcripts with FPKM higher than 5.000	0	2
Highest FPKM	1.826.80 (TR7339)	9.482.01 (TR5513)
Highest expression difference between outside and inside the block	69.70X (TR6118)	269.38X (TR6969)

Highly expressed genes (AVG + 3STD) between all libraries

The value of FPKM corresponding to AVG + 3STD was similar for the corresponding samples of the inside of blocks. The strain UFLA-LE6 showed a value of 6.2% superior to the strain UFLA-LE5. However, for the samples from surface, the strain UFLA-LE6 was 58% superior to the strain UFLA-LE5 (Table 5).

The number of highly expressed genes (HEG) was also similar between strains for samples from inside the blocks (Table 6). However, we observed a much higher number of HEG in the samples from the surface in the strain UFLA-LE5 (155), compared to the strain UFLA-LE6 (73), but most of this number (134) showed a FPKM value below 1,000 (Table 7). Only two transcripts from surface of UFLA-LE5 showed a FPKM value above 3,000, while for the strain UFLA-LE6 we observed 10 transcripts with FPKM above 3,000.

Table 5 - FPKM info per library. Average, median and standard deviation of the samples obtained from surface, inside the blocks and mycelium of UFLA-LE5 and UFLA-LE6 strains of *L. edodes*.

Strain	Sample	AVG	MEDIAN	STD	AVG + STD	AVG + 2STD	AVG + 3STD
UFLA LE5	I	28.30	3.75	163.74	192.03	355.77	519.51
	S	26.88	2.84	105.80	132.68	238.48	344.28
	M	31.00	3.94	193.27	224.28	417.55	610.83
UFLA LE6	I	32.45	4.36	173.13	205.58	378.71	551.84
	S	32.11	4.82	170.58	202.69	373.27	543.84
	M	34.79	4.16	250.16	284.95	535.11	785.28

* AVG: average; STD: standard deviation; S: surface: I: inside: M: mycelium.

Table 6 - Number of genes per FPKM limit.

Library	FPKM = 0	FPKM < 0.1	FPKM < 1	FPKM > Avg + STD	FPKM > Avg + 2STD	FPKM > Avg + 3STD
LE5_I	1844	1889	4370	312	861	66
LE5_S	2208	2285	4805	526	252	155
LE5_M	1585	1627	3945	249	123	69
LE6_I	1550	1595	3974	304	125	77
LE6_S	1566	1594	3796	271	123	73
LE6_M	1519	1558	3934	198	98	57

I: Samples from inside the block; S: Samples from surface the block; M: Samples from mycelium.

Table 7 - Number of highly expressed genes (HEG) according to the FPKM range.

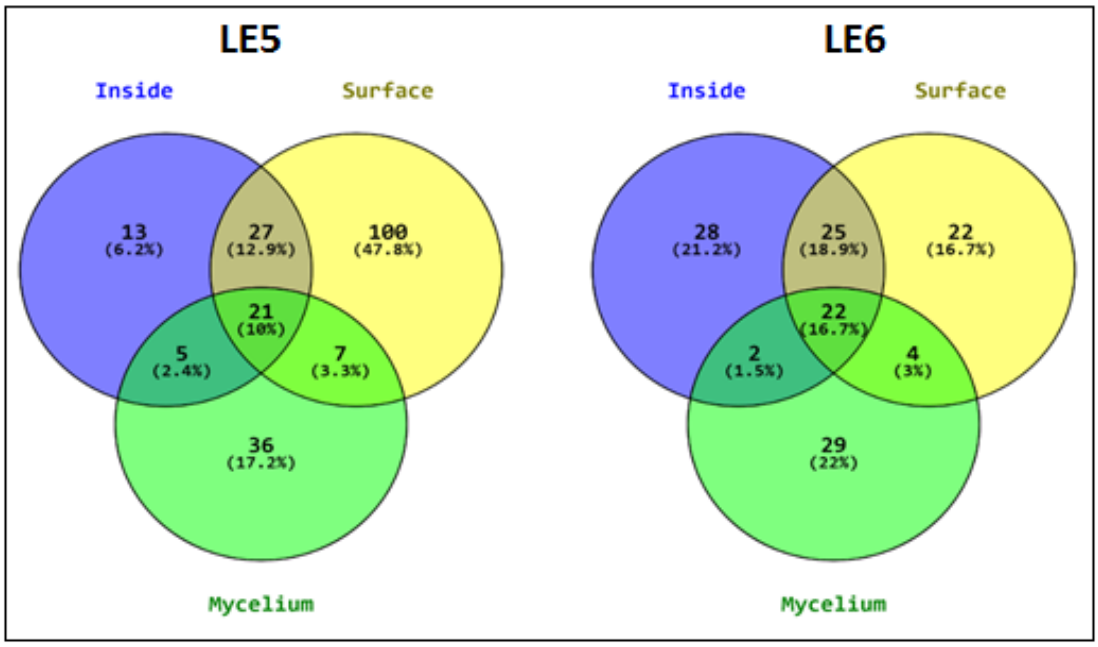
Strain	Sample	HE genes	Nº of genes with FPKM < 1000	Nº of genes with 1000 ≤ FPKM ≤ 3000	Nº of genes with 3000 ≤ FPKM ≤ 5000	Nº of genes with 5000 ≤ FPKM ≤ 10000	Nº of genes with FPKM ≥ 10000
UFLA LE5	I	66	48	13	2	2	1
	S	155	134	19	1	1	0
	M	69	26	35	3	4	1
UFLA LE6	I	77	47	24	3	2	1
	S	73	48	15	7	3	0
	M	57	13	32	6	5	1

I: interior; S: surface; M: mycelium.

Analyses of differentially expressed genes

The HEG were analyzed by the Venn´s diagram (Figure 1) aiming to find the genes expressed exclusively in each type of sample (surface, inside, and mycelium). For the strain UFLA-LE6 only 22 genes were highly expressed exclusively at the surface and 28 at the interior of the blocks, while for UFLA-LE5 a hundred of genes were highly expressed exclusively at the surface and only 13 at inside of the blocks. Comparing surface vs inside, we found 27 transcripts for UFLA-LE5 and 25 transcripts for UFLA-LE6, which were shared between both types of samples as HEG. Comparing surface vs interior vs mycelium, we found 21 transcripts for UFLA-LE5 and 22 transcripts for UFLA-LE6, shared as HEG between all types of samples.

Figure 1 - Venn diagram displaying the numbers of unique and shared expressed transcripts in the three samples (inside, surface and mycelium) from each strain (UFLA-LE5 and UFLA-LE6).



Selected genes from strains UFLA-LE6 and UFLA-LE5 for RT-qPCR

Considering the highly and differentially expressed genes in the samples from surface for both strains, we selected some genes in function of the FPKM value and difference of ratio between the values for surface and inside (S/I ratio), which are showed in the tables 8 and 9. Thus, these 13 transcripts were selected as the main candidate genes for the studies about gene expression in the browning process of *L.*

edodes. Some of these transcripts were highly expressed both at surface and at inside of the block but with a value of FPKM extremely high at surface and a S/I ratio higher than twice. No annotation was found for the transcript TR2915, however its FPKM value was the highest (8002.3). Other transcript was selected having a FPKM value below 1000, although with a S/I ratio extremely high (TR6969).

Identification and validation of reference genes for qPCR analysis

The GeNorm and NormFinder algorithms identified Fcf 2, ATPs 1 and Kin2 as the most stable genes along all the conditions assayed. Despite the large variation, the results are satisfactory considering that the samples were very heterogeneous, except for mycelium samples (Figure 2). Nevertheless, it was possible to find a pattern of these genes across samples of the two strains. As a consequence of these genes were selected as reference index for data normalization.

Table 8 - Selected genes of strain UFLA-LE6 with differential high expression only in the sample from exterior of the block (brown film) and their respective orthologs.

Strain UFLA-LE6						Strain UFLA-LE5				
Gene_id	**I**	**S**	**M**	**S/I**	**Functional annotation**	**Gene_id**	**I**	**S**	**M**	**S/I**
TR7601	59.1	571.1	28.3	9.66	Alcohol dehydrogenase	TR1226	269.3	397.8	23.9	1.48
TR2948	133.8	847.1	387.1	6.33	Thioredoxin reductase	TR2882	191.1	96.6	347.1	0.51
TR1421	417.8	988.8	319.4	2.37	3-oxoacyl CoA thiolase	TR4275	226.3	238.3	243.7	1.05
TR9170	483	2307.5	128.3	4.78	Cytochrome C	TR1573	373.7	1288.6	76.6	3.45
TR2915	2103.2	8002.3	18.6	3.80	NFA	TR2781	12.5	344.9	6.3	27.59

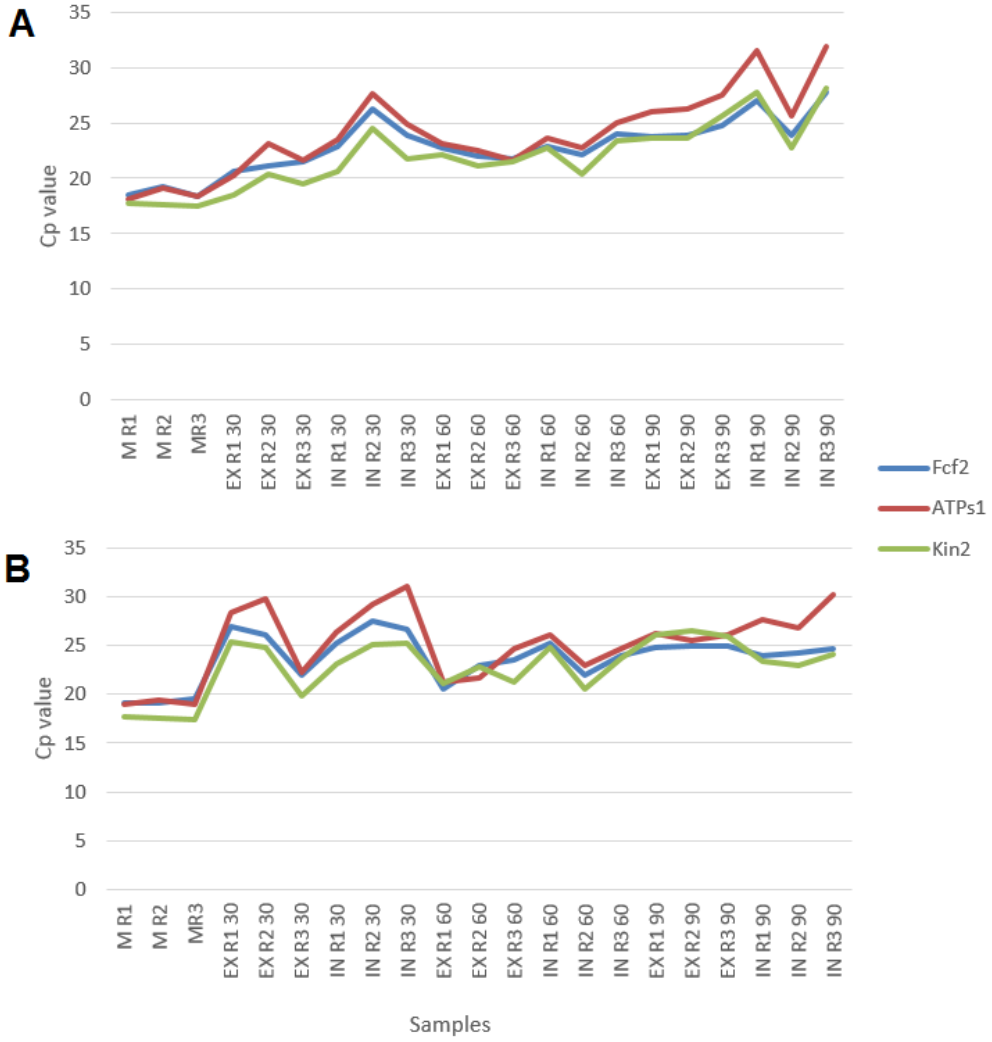
I: FPKM values from interior of the block; S: from surface of the block; M: from mycelium. S/I: ratio between FPKM values of the respective samples. NFA: non functional annotaion.

Table 9 - Selected genes of UFLA-LE5 strain with differential high expression in the sample from exterior of the block (brown film) and their respective orthologs.

Strain UFLA-LE5						Strain UFLA-LE6				
Gene_id	**I**	**S**	**M**	**S/I**	**Functional annotation**	**Gene_id**	**I**	**S**	**M**	**S/I**
TR2781	45.2	589.5	94.4	13.04	Aldehyde reductase	TR5952	56.9	59.8	84.3	1.05
TR1286	107.6	1094.1	69.6	10.17	Alcohol dehydrogenase	TR5513	1423.7	6550.4	44.6	4.6
TR579	229.7	1648.5	170.3	7.18	D-aspartate oxidase	TR533	667.3	5355.7	220.2	8.03
TR790	124.8	631.5	9.4	5.06	Uncharacterized conserved protein	TR6025	120.6	27.7	6.7	0.23
TR8749	157.1	792.9	19.3	5.05	Predicted hydrolases or acyltransferases	TR429	77.5	100.3	33	1.29
TR142	255.8	1057.2	274.3	4.13	3-oxoacyl-CoA thiolase	TR2963	286.7	328.3	299.3	1.15
TR7339	457.8	1959.3	9.7	4.28	Predicted haloacid-halidohydrolase and related hydrolases	TR107	46.7	81.4	11.9	1.74
TR5782	11.1	330.0	0.5	29.73	4-hydroxybenzoate polyprenyltransferase	TR6969	1.9	409.3	0.8	215.31

I: FPKM values from interior of the block; S: from surface of the block; M: from mycelium. S/I: ratio between FPKM values of the respective samples.

Figure 2 – Cp value efficiency-corrected of reference genes for all the samples. (A) UFLA-LE5 strain, (B) UFLA-LE6 strain.



Exploratory analysis of the expression profiles

Two genes were found in the UFLA-LE5 strain with statistical differences ($p < 0.05$). At 60 days aldehyde reductase gene was upregulated in the inside sample (1.58-fold) compared to the outside sample (-0.71-fold). However, CoA 1 gene expression was significantly downregulated in the outside sample at 90 days (Figure 3).

In the UFLA-LE6 strain three genes are statistically significant at $p < 0.05$. Alcohol dehydrogenase 1 presented an increase in downregulation from 60 to 90 days at inside samples (reaching -9.4-fold decrease), while the outside sample displayed a strong repression at 90 days (-0.13-fold). The second one was the gene that encodes a conservative protein, showing a decrease in upregulation in the outside

sample at 90 days (-0.11-fold). Interestingly, the NFA gene showed at 30 days a downregulation and a upregulation in inside and outside samples, respectively (Figure 4).

Some genes were statistically significant ($p < 0.05$) between the two strains. The first one was alcohol dehydrogenase 2, which exhibited at 60 days a similar upregulation in inside samples, but a decrease in UFLA-LE6 in the outside sample (1.13-fold). Another example is a gene that encodes a possible hydrolase or transferase, at 90 days the UFLA-LE6 strain displayed an upregulation decreased in inside samples while the outside sample had upregulation suppressed (-0.04-fold). Hydrolase gene in inside samples was upregulated in UFLA-LE5 strain (1.86-fold) and downregulated in UFLA-LE6 strain (-1.79-fold) at 90 days. Meanwhile, the outside sample ranged from -0.82 to 0.0 in UFLA-LE5 and UFLA-LE6 strains, respectively (Figure 5).

Figure 3 - Expression levels of inside and outside samples of UFLA-LE5 strain during brown film formation. (A) Aldehyde reductase gene, (B) 3-oxoacyl-CoA thiolase gene.

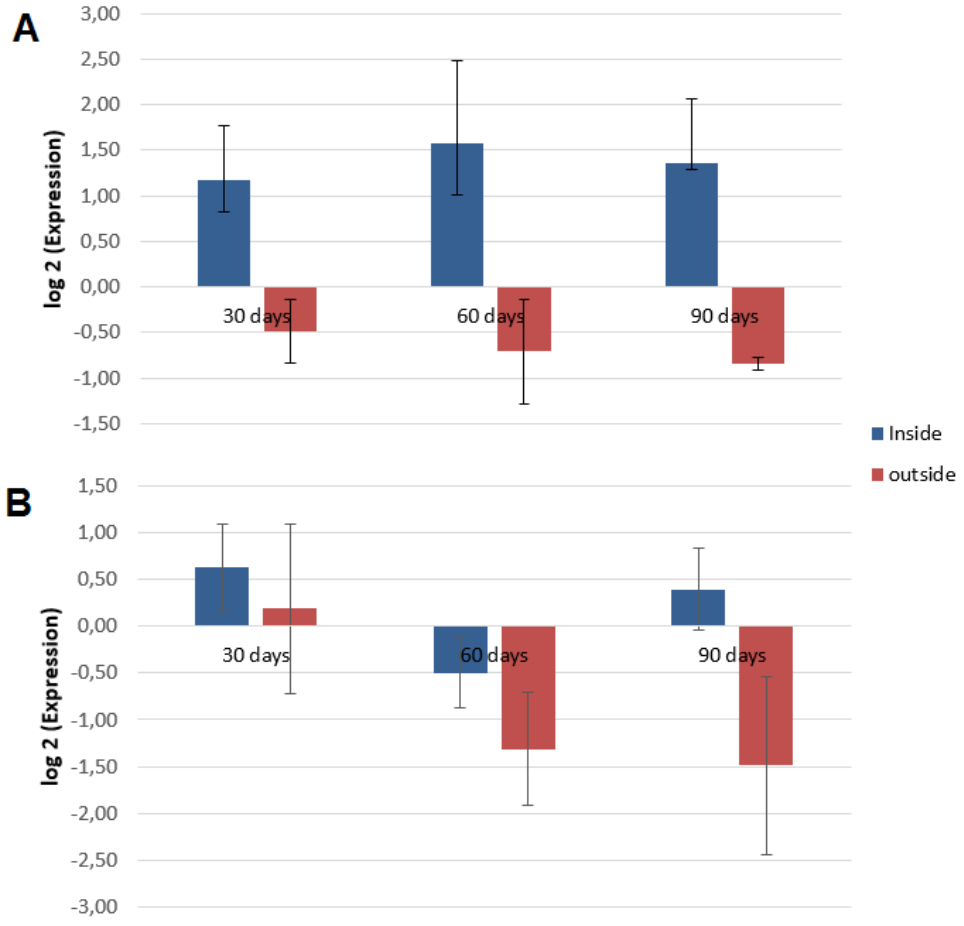


Figure 4 – Expression levels of inside and outside samples of UFLA-LE6 strain during brown film formation. (A) Alcohol dehydrogenase 1 gene; (B) Conserved protein gene; (C) NFA gene.

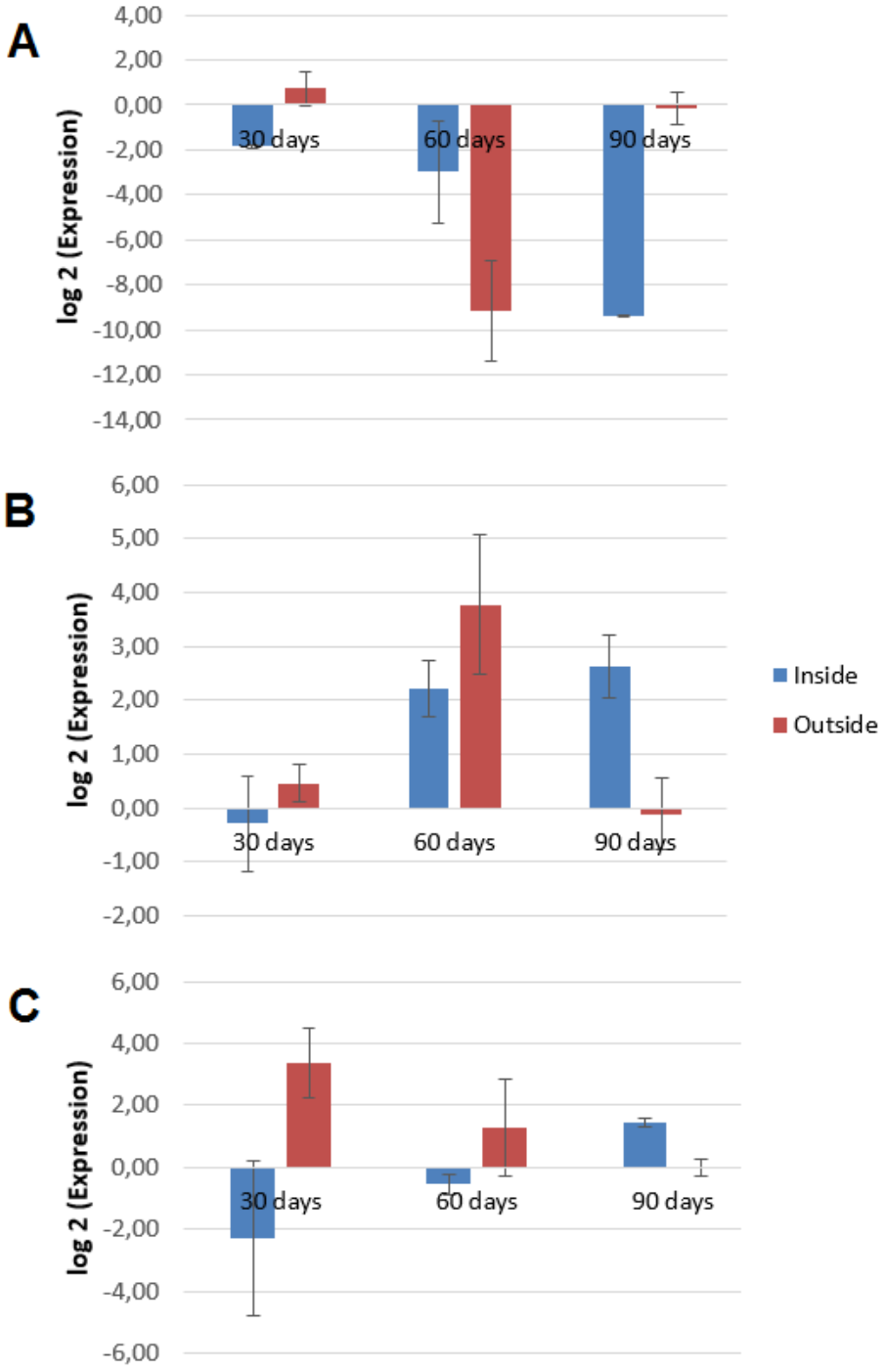
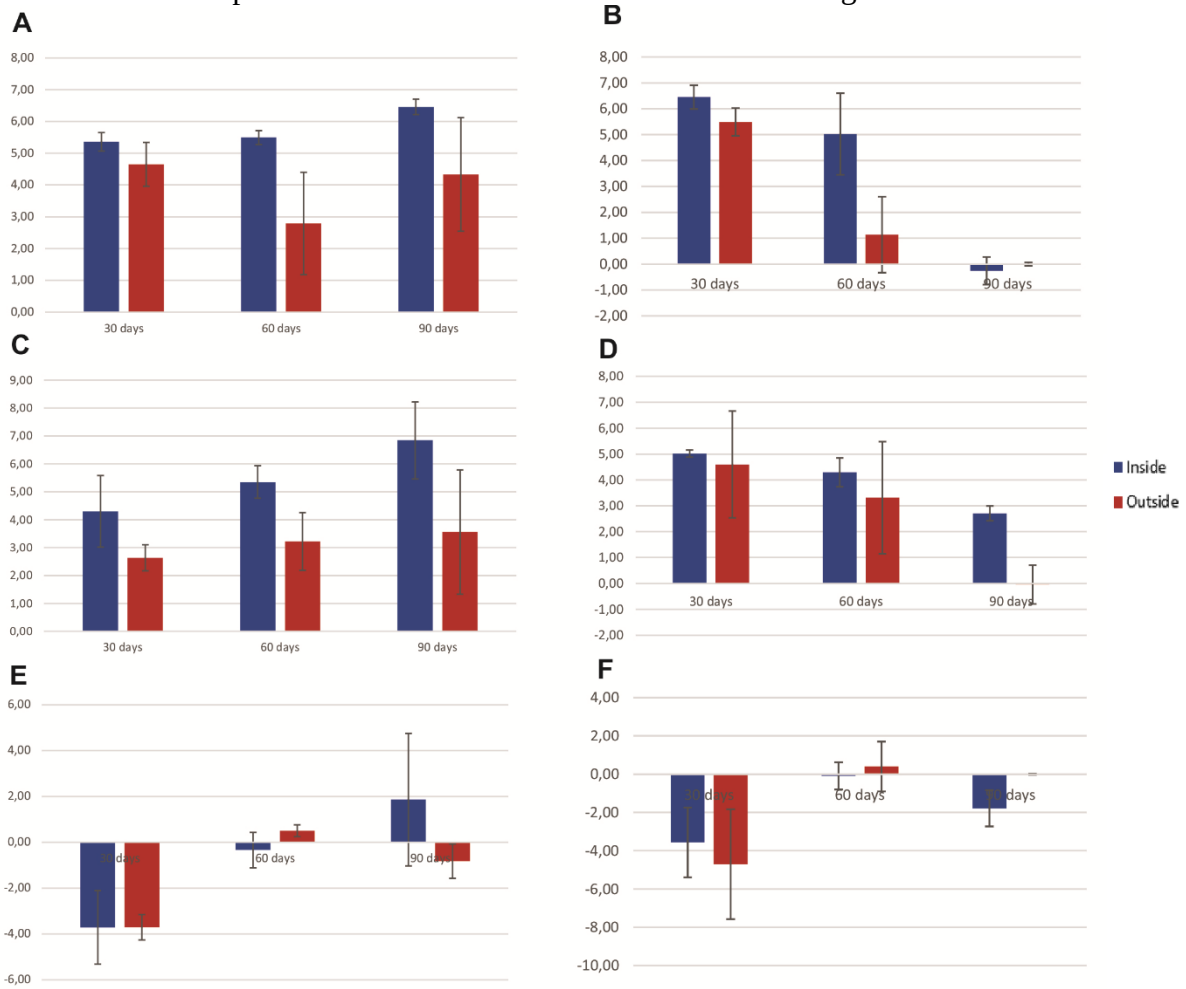


Figure 5 – Expression levels statistically significant ($p<0.05$) of inside and outside samples in UFLA-LE5 and UFLA-LE6 strain during brown film formation.



*left column: UFLA-LE5; right column: UFLA-LE6; A and B: alcohol dehydrogenase 2 gene; C and D: hydrolase/transferase gene; E and F: hydrolase gene.

Discussion

Some pioneering studies with *L. edodes* on this subject were made under conditions of synthetic culture media, instead conditions of real cultivation (TSIVILEVA et al., 2005; TSIVILEVA; PANKRATOV; NIKITINA, 2010; VETCHINKINA; POZDNYAKOVA; NIKITINA, 2008a; VETCHINKINA; POZDNYAKOVA; NIKITINA, 2008b; VETCHINKINA et al., 2008; VETCHINKINA; SOKOLOV; NIKITINA, 2008). Tang et al. (2013) reported the first study about transcriptional analysis aiming to find candidate genes for the browning. The authors used as approach the comparison between transcriptomes of fungal colonization under brown film-forming and non-brown

film-forming (light/dark regime and complete darkness, respectively). In function of this approach, the authors found upregulated candidate genes encoding proteins linked to light reception, light signal transduction pathways, and pigment formation.Other transcriptomic study was reported but using mycelium and with an interest at its medicinal properties or about genes involved in (ZHONG et al., 2013).

In this study, it was not find upregulated genes encoding the classical proteins or enzymes pointed out as important for brown film formation, like lectin, β-glucan, laccase and others found by Tang et al. (2013). This finding does not mean that these genes are not important, only that at this specific moment (spawn-run of 60 days), they are not highly expressed.

Another intriguing finding was the difference of the number of upregulated genes between strain UFLA-LE5 (less-productive) and strain UFLA-LE6 (productive). The strain UFLA-LE5 showed a much higher number of upregulated genes compared to UFLA-LE6 strain. At first, it seems to be contradictory; however, it may suggest a failure of the strain UFLA-LE5 to control the level of the genetic expression in order to guarantee only the most important genes to be highly expressed during the browning process. We may argue that so many upregulated genes in the strain UFLA-LE5 result in high energy utilization, contributing to a lower expression of the really necessary genes at that moment of substrate colonization.

None of the interest genes are directly related to light-induction or pigment production. However, we found two transcripts in strain UFLA-LE6 involved to the electron transfer chain (TR9170: Cytochrome c, TR6969: 4-hydroxybenzoate polyprenyltransferase), showing a much higher respiratory activity at surface compared to inside of the block (BRASSEUR et al., 1997). This result was expected considering the more limited oxygen availability compared to the oxygen availability at surface. In addition, oxygen is important to accelerate the browning, by the oxidation of phenolic compounds and pigment production (TANG et al., 2013).

We could argue that these genes may contribute for the difference between the strains about the brown film formation. However, we must consider that the strain UFLA-LE5 also showed some upregulated genes differentially expressed at surface, which were not found in the UFLA-LE6 strain. In this way, these genes could not be enough to ensure brown film formation, demonstrating that not all upregulated genes are necessarily essential or related to this process. Moreover, some genes may have an opposite effect, inhibiting instead stimulating it.

The transcript TR6969 in UFLA-LE6, showed a FPKM value of 409.3 and a S/I ratio of 215.3, which is extremely high, compared to all other transcripts. Its ortholog in the strain UFLA-LE5 showed a similar FPKM value (330.0) but a quite lower S/I ratio (29.7), but it may be considered a high S/I ratio,

compared to the other transcripts. In both orthologs (UFLA-LE6 and UFLA-LE5), the annotation for this transcript encodes 4-hydroxybenzoate polyprenyltransferase, an enzyme involved in the ubiquinone synthesis, which is an important element in the electron transfer chain. In addition, this enzyme could play additional roles, like as an antioxidant protective (UCHIDA et al., 2000). Given its extremely high S/I ratio, with negligibly FPKM at inside and mycelium, we may hypothesize that this enzyme may be very important in a substrate rich in lignin, where many oxidative reactions take place.

The transcript TR533 was upregulated, with a FPKM value of 5,355.7 in UFLA-LE6 and encodes for a D-aspartate oxidase. This annotation came from Actinobacteria (*Mycobacterium tuberculosis* complex), but this enzyme has been described in many species of fungi, including basidiomycetes. There are many questions about its physiological function, including a suggestion that the enzyme did not have a physiological significance (OHNISHI; MACLEOD; HOROWITZ, 1962). The D-aspartate oxidase catalyzes the oxidative deamination of D-aspartate, resulting in oxaloacetate, ammonia and hydrogen peroxide. Basically, it is supposed that this enzyme has the main function of to make D-amino acids available for cell metabolism (POLLEGIONI et al., 2007). In addition, the enzyme has a proposed function of catabolic use of D-amino acids as carbon, nitrogen, and energy source in *E. coli*, besides a protective role against the toxic effects of aromatic D-amino acids, in *Neurospora crassa* (POLLEGIONI et al., 2007; POLLEGIONI et al., 2008). The substrate for shiitake cultivation used in this work is very rich in protein sources, like rice and wheat bran and corn meal. Therefore, it is expected to happen protein degradation, resulting in a high concentration of different kinds of amino acids. Considering this scenario, the D-aspartate oxidase may have an important function in the degradation of amino acids, not only for the cell metabolism but also eliminating excess of aromatic D-amino acids. Further studies using substrates poorly in protein sources compared to the richer ones will be necessary to confirm this hypothesis.

The transcript TR2915 was the most expressive one, with the highest FPKM value, considering the differentially expressed genes. This gene was upregulated both at surface and inside of the block and downregulated at mycelium. Its FPKM value was 8,002.3 and 2,103.2 at surface and inside of the block, respectively, with a S/I ratio of 3.80. Unfortunately, there is no annotation about this transcript, consequently, we do not know about its probably function during the browning process. Therefore, this transcript requires some analysis to find a putative protein it encodes.

Finally, the transcript TR5513, encoding an alcohol dehydrogenase, was upregulated at both, surface and inside of the block. However, we observed a S/I ratio of 4.6 and a FPKM value of 6,550.4 at surface, which is much higher than other upregulated transcripts discussed before. The high value of FPKM observed inside the block is an evidence that the alcohol dehydrogenase is very important for both

substrate environments, surface and inside. On the other hand, the differential expression between surface and inside is also an evidence that the enzyme activity is even more important for the browning process. This difference suggests a need of a stronger enzyme activity to guarantee the favorable conditions for the browning process.

The alcohol dehydrogenases are a large group of oxidoreductases which catalyze the reversible oxidation of alcohol to aldehyde or ketone. The enzyme activity results in the reduction of NAD^+ or $NADP^+$. In *Saccharomyces cerevisiae*, the alcohol dehydrogenase is very well known as an important enzyme in sugar metabolism (DE SMIDT; DU PREEZ; ALBERTYN, 2008). Different isozymes act for different functions like as to regenerate the glycolytic NAD^+, restoring the redox balance, through the production of ethanol from acetaldehyde, or the opposite, converting ethanol to acetaldehyde, with the reduction of NAD^+. However, this enzyme activity is not exclusive to yeasts, but it has been reported to filamentous fungi too. The fermentation may be a necessary metabolic mechanism under limited levels of oxygen (GRAHL et al., 2011). Under this condition, the fungus needs fermentation to replace NAD^+ and keeps producing ATP through glycolysis. In addition, De Smidt, Du Preez and Albertyn (2008) suggested that this enzyme may capacitate the yeasts to live in ligninolytic environments.

The substrate for shiitake cultivation is very rich in lignin and cellulose, considering that it is a sawdust-based substrate. Therefore, we must considerate the possibility that the alcohol dehydrogenase has as physiological function the utilization of the products derived from lignin degradation. However, taking in account the very high FPKM value for this gene, it is more probably that this enzyme is necessary for a more critical function.

During all time, the spawn-run and browning were carried out with the substrate inside the plastic bag, where the oxygen availability probably got more and more limited, even having a window for gaseous exchange. It is interesting to note that inside the block, the FPKM value observed was lower than at surface, even being upregulated with an expression extremely high compared to its reference value (1,423.7). Therefore, the enzyme activity was necessary at high level inside the block, where it is supposed to have a more limited oxygen availability, but it was even more necessary at the surface. The highest genetic expression for this gene at surface, in despite of having more oxygen availability, is an evidence that this enzyme plays a vital function for the browning process.

Several housekeeping genes, such as actin1, gapdh1 have been widely used as internal standards for the normalization of RT-qPCR data. During the last decade, some studies have revealed the instability of housekeeping gene expression, although they can also be acceptable internal standards under certain conditions (PAOLACCI et al., 2009; FAN et al., 2013; CHENG et al., 2013). This fact is evidence that

gene expression stability varies with the gene, species, tissue, and culture conditions used (JAIN et al., 2006; JIAN et al., 2008). Thus, our results reinforce the fact that reference genes should not be chosen on the unique basis of suitability in other organisms. Therefore, if no data for a given species are available, experimental validation should always be the first step in RT-qPCR expression studies. In this sense, geNorm and NormFinder are the methods commonly used for analyzing the stability of reference gene panels.

In general, the results of RT-qPCR that were statistically significant ($p<0.05$) showed us a very similar behavior of the UFLA-LE6 strain. The alcohol dehydrogenase, conservative protein, hydrolase and transferase genes always exhibited a decrease in expression on the surface at 60 or 90 days. This profile can be explained by the speed and stability with which the brown film is formed in this strain. Thus, genes that previously appeared to have a certain function in that process, at the end of cultivation they are no longer needed. Only the NFA gene showed a distinct behavior in the UFLA-LE6 strain, where its expression in the outside sample was upregulated at 30 days. Due to the lack of this gene annotation, we can conclude that its action occurs at the beginning of the culture cycle, and may be an enzyme that degrades primary compounds of the substrate helping mycelial growth.

In conclusion, advances in sequencing technologies are increasing the affordability of whole-transcriptome analyses. However, exploratory analyses of transcriptomes using RNA-seq are becoming frequent and the need for biological replicates to achieve proper statistical robustness makes confirmatory analyses very expensive. In addition, the results of differential expression analyses using RNA-seq are influenced by data postprocessing and the statistical test/package used for the analysis (RAPAPORT et al., 2013). In this sense, since the publication of guidelines on the minimum information for publication of quantitative real-time PCR experiments (MIQE guidelines) for RT-qPCR assays (BUSTIN et al., 2009) and their increasing adoption by the scientific community, have becoming this technique very robust and reproducible. Moreover, RT-qPCR is considered the gold standard of expression analyses, due to wide amplification rangeand the small amount of sample needed. In this context, RT-qPCR and RNA-seq will probably evolve as complementary approaches for studies aimed at answering the biological questions underlying changes in differential expression (CASTANERA et al., 2015).

Acknowledgements

The FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) are acknowledged for financial support.

References

ANDERSEN, C. L.; JENSEN, J. L.; ØRNTOFT, T. F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. **Cancer research**, v. 64, n. 15, p. 5245-5250, 2004.

BRASSEUR, G. et al. The nuclear ABC1 gene is essential for the correct conformation and functioning of the cytochrome bc1 complex and the neighbouring complexes II and IV in the mitochondrial respiratory chain. **European journal of biochemistry**, v. 246, n. 1, p. 103-111, 1997.

BUSTIN, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. **Clinical chemistry**, v. 55, n. 4, p. 611-622, 2009.

CASTANERA, R. et al. Validation of reference genes for transcriptional analyses in *Pleurotus ostreatus* using RT-qPCR. **Applied and environmental microbiology**, v. 81, n. 12, p. 4120-4129, 2015.

CHENG, D. et al. Validation of reference genes in Solenopsis invicta in different developmental stages, castes and tissues. **PLoS One**, v. 8, n. 2, p. e57718, 2013.

DE SMIDT, O.; DU PREEZ, J. C.; ALBERTYN, J. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. **FEMS yeast research**, v. 8, n. 7, p. 967-978, 2008.

DIAS, E. S. Mushroom cultivation in Brazil: challenges and potential for growth. **Ciência e Agrotecnologia**, v. 34, n. 4, p. 795-803, 2010.

FAN, C. et al. Selection of reference genes for quantitative real-time PCR in bamboo (Phyllostachys edulis). **PloS one**, v. 8, n. 2, p. e56573, 2013.

GRAHL, N. et al. *In vivo* hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. **PLoS pathogens**, v. 7, n. 7, p. e1002145, 2011.

HELLEMANS, J. et al. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. **Genome biology**, v. 8, n. 2, p. 19, 2007.

HIGUCHI, R. et al. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. **Nature Biotechnology**, v. 11, n. 9, p. 1026, 1993.

JAIN, M. et al. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. **Biochemical and biophysical research communications**, v. 345, n. 2, p. 646-651, 2006.

JIAN, B. et al. Validation of internal control for gene expression study in soybean by quantitative real-time PCR. **BMC molecular biology**, v. 9, n. 1, p. 59, 2008.

KÜES, U.; LIU, Y. Fruiting body production in basidiomycetes. **Applied microbiology and biotechnology**, v. 54, n. 2, p. 141-152, 2000.

MA, L. et al. A critical role for the loop region of the basic helix–loop–helix/leucine zipper protein Mlx in DNA binding and glucose-regulated transcription. **Nucleic acids research**, v. 35, n. 1, p. 35-44, 2006.

OHNISHI, E.; MACLEOD, H.; HOROWITZ, N. H. Mutants of *Neurospora* deficient in D-amino acid oxidase. **Journal of Biological Chemistry**, v. 237, n. 1, p. 138-142, 1962.

PAOLACCI, A. R. et al. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. **BMC molecular biology**, v. 10, n. 1, p. 11, 2009.

PFAFFL, M. W. A new mathematical model for relative quantification in real-time RT–PCR. **Nucleic acids research**, v. 29, n. 9, p. 45, 2001.

PFAFFL, M. W.; HORGAN, G. W.; DEMPFLE, L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. **Nucleic acids research**, v. 30, n. 9, p. 36, 2002.

PFAFFL, M. W. **Quantification strategies in real-time PCR.** p. 87–112. In: Bustin, S. A. A-Z of quantitative PCR. International University Line, La Jolla, CA, 2004.

POLLEGIONI, L. et al. Physiological functions of D-amino acid oxidases: from yeast to humans. **Cellular and molecular life sciences**, v. 64, n. 11, p. 1373-1394, 2007.

POLLEGIONI, L. et al. Properties and applications of microbial D-amino acid oxidases: current state and perspectives. **Applied microbiology and biotechnology**, v. 78, n. 1, p. 1-16, 2008.

RAPAPORT, F. et al. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. **Genome biology**, v. 14, n. 9, p. 3158, 2013.

ROYSE, D. J. Cultivation of shiitake on natural and synthetic logs. **College of Agricultural Sciences, Agricultural Research and Cooperative Extension. The Pennsylvania State University.** 2009.

RUIJTER, J. M. et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. **Nucleic acids research**, v. 37, n. 6, p. 45, 2009.

SPIESS, A. N.; FEIG, C.; RITZ, C. Highly accurate sigmoidal fitting of real-time PCR data by introducing a parameter for asymmetry. **BMC bioinformatics**, v. 9, n. 1, p. 221, 2008.

TANG, L. et al. Transcriptome analysis of candidate genes and signaling pathways associated with light-induced brown film formation in *Lentinula edodes*. **Applied Microbiology & Biotechnology**, Berlin, v. 97, n. 11, p. 4977-4989, June 2013.

TICHOPAD, A. et al. Standardized determination of real-time PCR efficiency from a single reaction set-up. **Nucleic acids research**, v. 31, n. 20, p. 122, 2003.

TSIVILEVA, O. M. et al. Effect of media components on the mycelial film formation in submerged culture of *Lentinus edodes* (Shiitake). **Food Technology and Biotechnology**, v. 43, n. 3, p. 227-234, 2005.

TSIVILEVA, O. M.; PANKRATOV, A. N.; NIKITINA, V. E. Extracellular protein production and morphogenesis of *Lentinula edodes* in submerged culture. **Mycological progress**, v. 9, n. 2, p. 157-167, 2010.

UCHIDA, N. et al. Phenotypes of fission yeast defective in ubiquinone production due to disruption of the gene for p-hydroxybenzoate polyprenyl diphosphate transferase. **Journal of bacteriology**, v. 182, n. 24, p. 6933-6939, 2000.

VANDESOMPELE, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome biology**, v. 3, n. 7, p. research0034. 1, 2002.

VAULONT, S.; VASSEUR-COGNET, M.; KAHN, A. Glucose regulation of gene transcription. **Journal of Biological Chemistry**, 2000.

VETCHINKINA, E. P.; POZDNYAKOVA, N. N.; NIKITINA, V. E. Laccase and lectin activities of intracellular proteins produced in a submerged culture of the xylotrophic basidiomycete *Lentinus edodes*. **Current microbiology**, v. 57, n. 4, p. 381-385, 2008a.

VETCHINKINA, E. P.; POZDNYAKOVA, N. N.; NIKITINA, V. E. Enzymes of the xylotrophic basidiomycete *Lentinus edodes* F-249 in the course of morphogenesis. **Microbiology**, v. 77, n. 2, p. 144-150, 2008b.

VETCHINKINA, E. P. et al. Activity of *Lentinus edodes* intracellular lectins at various developmental stages of the fungus. **Applied biochemistry and microbiology**, v. 44, n. 1, p. 66-72, 2008.

VETCHINKINA, E. P.; SOKOLOV, O. I.; NIKITINA, V. E. Intracellular lectins of *Lentinus edodes* at various developmental stages of the fungus. **Microbiology**, v. 77, n. 4, p. 441-445, 2008.

ZHONG, M. et al. De novo characterization of *Lentinula edodes* C91-3 transcriptome by deep Solexa sequencing. **Biochemical and biophysical research communications**, v. 431, n. 1, p. 111-115, 2013.

ZIED, D. C. et al. Selection of strains for shiitake production in axenic substrate. **World Journal of Microbiology and Biotechnology**, v. 32, n. 10, p. 168, 2016.