Brazilian Journal of Microbiology (2001) 32:170-175
ISSN 1517-8382

ANALYSES OF GENETIC VARIABILITY IN *LENTINULA EDODES* THROUGH MYCELIA RESPONSES TO DIFFERENT ABIOTIC CONDITIONS AND RAPD MOLECULAR MARKERS

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Submitted: November 08, 2000; Returned to authors for corrections: March 12, 2001; Approved: June 2001

ABSTRACT

The growth of thirty-four *Lentinula edodes* strains submitted to different mycelial cultivation conditions (pH and temperature) was evaluated and strain variability was assessed by RAPD molecular markers. The growth at three pH values (5, 6 and 7) and four different temperatures (16, 25, 28 and 37ºC) was measured using the *in vitro* mycelial development rate and water retention as parameters. Mycelial cultivation was successful at all pH tested, while the ideal temperature for mycelial cultivation ranged between 25 and 28ºC. The water content was lower in strains grown at 37ºC. Among 20 OPA primers (Operon Technologies, Inc.) used for the RAPD analyses, seventeen presented good polymorphism (OPA01 to OPA05, OPA07 to OPA14, OPA17 to OPA20). The clustering based on similarity coefficients allowed the separation of strain in two groups with different geographic origins.

Key words: edible mushroom, RAPD, mycelial growth

INTRODUCTION

The edible mushrooms have been used as a food alternative for more than 2000 years in Europe and Asia, where they were cultivated for consumption under primitive conditions (6). The shiitake [*Lentinula edodes* (Berk.) Pegl.] stands out among the species cultivated in the past and today it is produced on tree logs or sawdust (for example, eucalyptus, oak, mango, etc.). *L. edodes* is the third most important edible mushroom in the world in terms of total production (3,4). Shiitake mushrooms develop as saprophytes on tree logs and form fruit bodies at low temperature (15 – 20ºC) and high humidity levels. These mushrooms grow in the wild in China, Japan, Korea, the Himalayas, the Philippines, Papua New Guinea and in the north of Thailand (1,10). The scientifically proven therapeutic qualities of shiitake have raised the interest of researchers to isolate new active compounds from this mushroom. Although it is an age-old crop in Asian countries, its cultivation is recent in Brazil (1990s) and is mainly concentrated in the south and southeast areas of the country. In these regions, the shiitake produced is used for human consumption.

Shiitake cultivation can be divided into two stages: incubation and harvesting (8). During incubation, the mycelia secrete extra-cellular enzymes that act on substrate to form components that are then absorbed by the mycelia and used as source of raw material and energy for the metabolism. In the fructification or harvesting phase, the fungus begins to project the mushrooms in response to an external signal, which is generally a physical and/or a heat stress.

Little information is available on the behavior of *L. edodes* strains cultivated in tropical countries. This study was carried out on 34 *L. edodes* strains to characterize their development response to different pH and temperature conditions, and to analyze their genetic variability using RAPD (Random Amplified Polymorphic DNA) molecular markers. This type of investigation provides essential information for shiitake strain breeding aiming high quality production. The present studies were focused on the *L. edodes* incubation phase, where the mycelia response to abiotic factors is directly related to the mycelial development rate.

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MATERIALS AND METHODS

Strains

34 dikaryotic *L. edodes* strains were used (Group A: 46-48; 50-52; 56-62; 66; 72; 74-76; 79; 102; Group B: Assaí; Ide-A, C; K1-4; Le1-5; Le10 and Curitiba). The strains in Group A came from the Instituto de Botânica (São Paulo/SP/Brazil) and those in group B belong to the *L. edodes* strains bank of the Laboratório de Genética de Fungos of the Universidade Estadual de Londrina (Londrina/PR/Brazil). The strains were maintained on Potato Dextrose Agar medium (PDA – Biobrás).

Assessment of mycelial development in different pH

Each one of the 34 *L. edodes* strains was transferred (three 10mm discs of agar containing mycelia) to Erlenmeyer flasks containing 50mL of potato broth at pHs 5, 6 and 7 and incubated at 25ºC for 15 days. After this period the mycelia were filtered in a vacuum pump, washed in distilled water, weighed (moist weight – MW) and dehydrated at 50ºC to obtain the dry weight (DW). The mycelial water content (MWC) was calculated according to:

MWC = 100 – [(DW/MW) x 100], where,

MWC = mycelial water content

DW = mycelial dry weight

MW = moist mycelial weight

Mycelial development was assessed from the dry weight of each individual strain.

Mycelial development assessment at different temperatures

Each one of the 34 *L. edodes* strains were transferred (three 10mm discs of agar containing mycelia) to Erlenmeyer flasks containing 50mL of potato broth at pH 6.8 and incubated at 16, 25, 28 and 37ºC for 15 days. After this period the mycelia were filtered and washed in a vacuum pump. The mycelia were weighed and dehydrated at 50ºC to obtain the dry weight (DW), and the mycelial water content (MWC) was calculated by the formula described above. A randomized complete block design with four replications was employed for both experiments: mycelial development in different pH and different temperatures. The mean comparison was done by the Tukey test at 5% probability.

Nucleic acid extraction

The *L. edodes* mycelia were cultivated in 50mL of potato broth for 15 days at 25ºC, filtered in a vacuum pump, washed in autoclaved distilled water, weighed and squashed in liquid nitrogen. For each gram of squashed mycelia, 4mL of extraction buffer pH 8.0 were added (Sigma Chemical Co.: 200mM Tris-HCl, 250mM NaCl, 25mM EDTA, 1% SDS (p/v), 1µL of β-Mercaptoethanol for each milliliter of buffer, ultra pure water). The material was placed in a water bath (65ºC) for 15 minutes. It was then washed successively with phenol, phenol + chloroform (1:1) and chloroform + octanol (24:1) – Sigma Chemical Co. Each washing was followed by centrifugation at 4,000rpm for 15 minutes (IEC, Centra CL2) and the precipitate was discarded. The nucleic acids were precipitated with isopropanol (-20ºC, Sigma Chemical Co.), inverted for 30 minutes, centrifuged at 8,000rpm for 5 minutes (Eppendorf Centrifuge 5402), three times washed with 70% ethanol (Labsynth) and re-suspended in TE buffer (10mL Tris 1M pH 8, 2mL EDTA 0.5M pH 8, 988mL ultra pure water). The tubes were kept under refrigeration (4ºC overnight). For each 100µL of nucleic acid solution, 10µL of RNase (10mg/mL, Boehringer Mannhein) was added and the mixture placed in a water bath for 10 minutes at 65ºC and then washed with phenol + chloroform (1:1), centrifuged at 3,000rpm for 5 minutes. Chilled ethanol was then added to the supernatant for DNA re-precipitation. Aliquots of 5µL of DNA solution were placed in 0.8% agarose (Sigma Chemical Co.) gel for quantification by comparison with a quantifying standard (control). A spectrophotometer was used to determine the A_{260}/A_{280} relationship.

RAPD reaction

The kit A primers from Operon Technologies (OPA01 to OPA20) were used and their sequences are shown in Table 1. The thermocycler (Perkin Elmer Gene Amp 9.600) was used in the amplification reactions characterized by the following steps: 95°C (1 minute) + 35 x 94ºC (10 seconds), 36ºC (1 minute), 72ºC (2 minutes) + 72ºC (7 minutes). The negative control consists

Table 1. Specific base sequences and molecular weight of the Operon Technologies primers.

Primer	Sequence (5'→3')	Molecular Weight
OPA 01	CAGGCCCTTC	2955
OPA 02	TGCCGAGCTG	3035
OPA 03	AGTCAGCCAC	2988
OPA 04	AATCGGGCTG	3059
OPA 05	AGGGGTCTTG	3090
OPA 06	GGTCCCTGAC	2995
OPA 07	GAAACGGGTG	3108
OPA 08	GTGACGTAGG	3099
OPA 09	GGGTAACGCC	3044
OPA 10	GTGATCGCAG	3059
OPA 11	CAATCGCCGT	2979
OPA 12	TCGGCGATAG	3059
OPA 13	CAGCACCCAC	2933
OPA 14	TCTGTGCTGG	3041
OPA 15	TTCCGAACCC	2939
OPA 16	AGCCAGCGAA	3037
OPA 17	GACCGCTTGT	3010
OPA 18	AGGTGACCGT	3059
OPA 19	CAAACGTCGG	3028
OPA 20	GTTGCGATCC	3010

of a sample containing only PCR buffer, primer, dNTPs, Taq polymerase and ultra pure water. The electrophoresis was performed in 1.2% agarose gels, that was stained in 10% ethidium bromide solution and bands were observed in an UV transilluminator (UVP). The data obtained by RAPD were analysed by the construction of a agreement matrix, by scoring the presence (1) or abscence (0) of a specific band on the gel for each one of the 17 primers tested. These analyses were carried out considering a total number of 110 polymorphic bands and the program used was Statistica 5.0 version (StatSoft, Inc.).

RESULTS AND DISCUSSION

Assessment of *L. edodes* mycelial development in three pHs

The influence of the culture medium pH was assessed on the *L. edodes* mycelial development and on the mycelial water content (MWC) of *L. edodes* strains cultivated under submerged culture at 25ºC for 15 days. The strains developed well at the three tested pHs for fungus growth and, therefore, any of these pHs can be used for *L. edodes* cultivation in liquid medium. Fig. 1 shows the behavior of eight strains (the most contrasting of the 34 tested) in the test. Tukey test ($p \leq 0.05$) was used. No MWC differences were observed between the strains within the same pH and, also, no significant variation in any strain was detected in the analyses done in the three pHs (Fig. 2). Fungi develop in optimum pH ranges, which are related to enzymatic systems, essential vitamin entry in the cell, surface metabolic reactions and mineral capture (5). Some fungi, however, are able to adjust to the pH of the medium, optimizing it for their better development.

No significant differences were observed among the pHs tested for *L. edodes* development, probably because the mycelia adapted to the medium pH. Khan *et al*. (9) observed a pH reduction along the vegetative development of *L. edodes* obtaining pH 5 as optimum for initial *L. edodes* mycelial growth, and a stabilization at pH close to 3.3, which would indicate the fungus passage from the first development phase (incubation phase) to the second (harvesting phase) (7,8). The results showed here did not detect significant pH effect on the fungal development rate.

Mycelial development assessment at different temperatures

The temperatures of 25ºC and 28ºC were the most suitable for *in vitro L. edodes* growth. The temperature of 37ºC was extremely unfavorable, while 16ºC it just allowed the development of the strains K2 and Le3, indicating an interesting characteristic for cultivation of these strains in regions with a cooler climate (16ºC) (Fig. 3). No significant MWC difference was observed among the strains cultivated at the same temperatures (Fig. 4). In practice, the best strains for best quality seem to be those which require lower temperatures to reach the fructification phase (8-16ºC). However strains should be found for fructification at high temperatures to meet the needs of the mushroom market in countries with a tropical climate (2). As mycelial growth and fructification are different stages of the shiitake life cycle, the ideal temperature for one is not always the ideal for the other. Thus, the temperature effect on the two development stages should be considered, and strains that can grow under various temperatures should be selected to meet the grower needs.

RAPD molecular markers analyses of the intraspecific variability

Genetic variability for mushroom yield and quality is necessary for breeding higher yielding and better quality of the

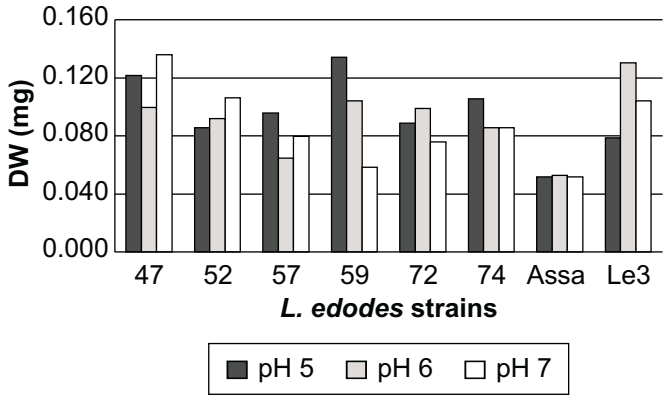

Figure 1. Mycelial Dry Weight (DW) of the eight most contrasting *L. edodes* strains cultivated in PDA medium at different pHs (5, 6 and 7), 25ºC for 15 days. The pH treatments for DW were not significantly different by the Tukey test at 5% probability.

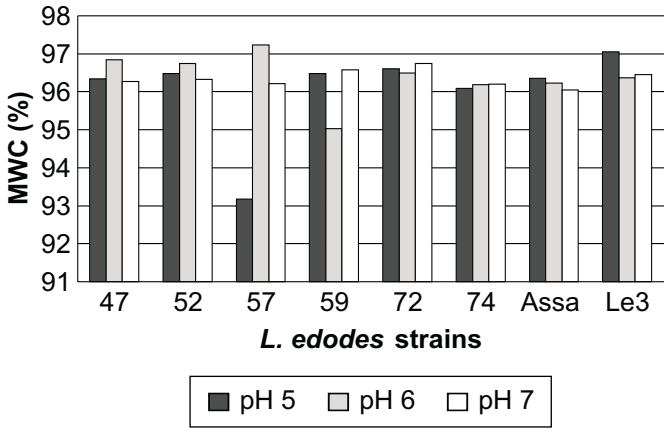

Figure 2. Mycelial Water Content (MWC) of the eight most contrasting *L. edodes* strains cultivated at three different pHs (5, 6 and 7), 25ºC for 15 days. The pHs treatments for MWC were not significantly different by the Tukey test at 5% probability.

shiitake. Mushroom strains may differ for traits that can be recombined through crosses in conventional genetic breeding, or they can be submitted to protoplast fusion techniques or to transformation with cloned genes using recombinant DNA technology (2). Thus, the importance of assessing the genetic variability of the strains deposited in a germplasm bank. RAPD data (Fig. 5) shows that the technique can be used as a routine methodology for genotype identification (type) in *L. edodes*. Three primers (OPA06, OPA15 and OPA16) did not amplify the *L. edodes* DNA in the RAPD amplification reaction. This result

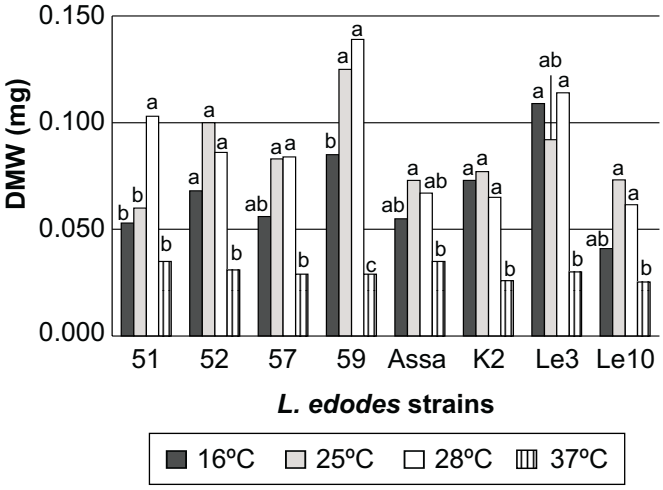

Figure 3. Mycelial dry weight (DW) of the eight most contrasting *L. edodes* strains cultivated at different incubation temperatures (16, 25, 28, 37ºC) in PDA, pH 6.8 for 15 days. Strains K2 and Le3 satisfactorily developed at 16ºC indicating adaptation to reduced temperatures (winter strains). The temperatures treatments for DW were significantly different by the Tukey test at 5% probability. Means followed by the same letter did not differ statistically.

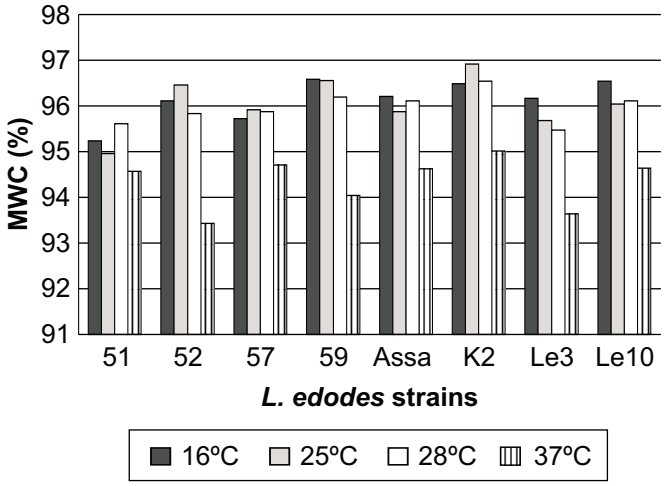

Figure 4. Mycelial Water Content (MWC) of the eight most contrasting *L. edodes* strains cultivated at four incubation temperatures (16, 25, 28 and 37ºC) in PDA media, pH 6.8 for 15 days. The temperatures treatments for MWC were not significantly different by the Tukey test at 5% probability.

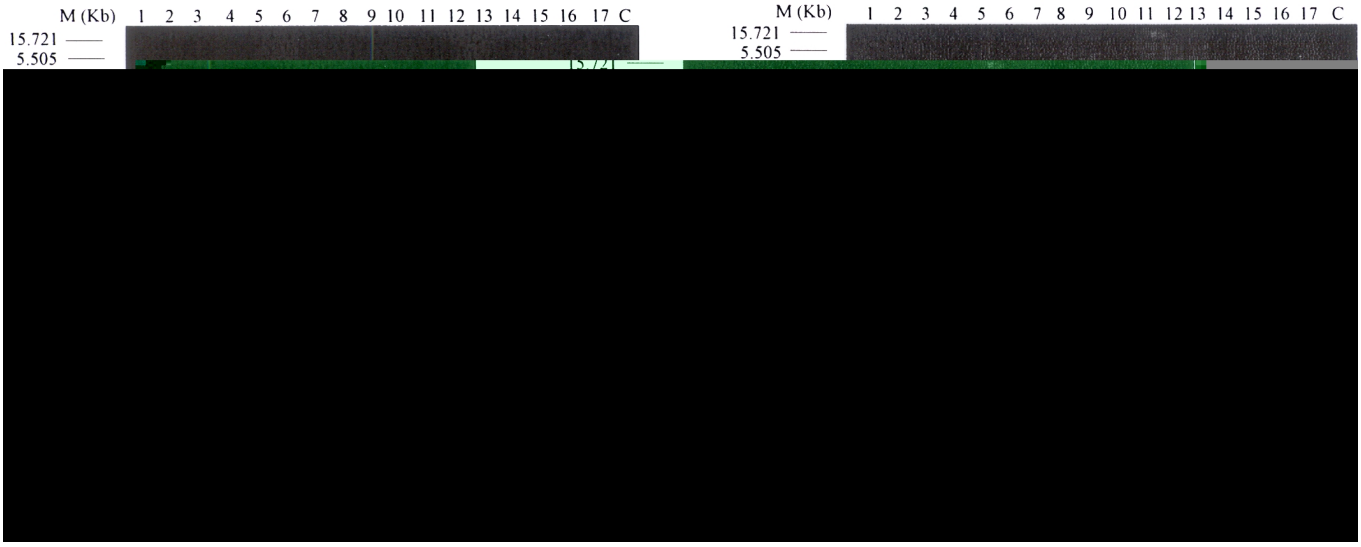

Figure 5. *L. edodes* amplification products in 1.2% agarose gels, using the RAPD primers OPA08 (left) and OPA10 (right). Key: (M) Molecular weight; (C) Negative control and *L. edodes* strains: (1) 46; (2) 47; (3) 48; (4) 50; (5) 51; (6) 52; (7) 56; (8) 57; (9) 58; (10) 59; (11) 60; (12) 61; (13) 62; (14) 66; (15) 72; (16) 74; (17) 75; (18) 76; (19) 79; (20) 102; (21) Assaí, (22) Ide-A; (23) Ide-C; (24) K2; (25) K3; (26) K4; (27) Le1; (28) Le2; (29) Le3; (30) Le4; (31) Le5; (32) Le10; (33) Curitiba.

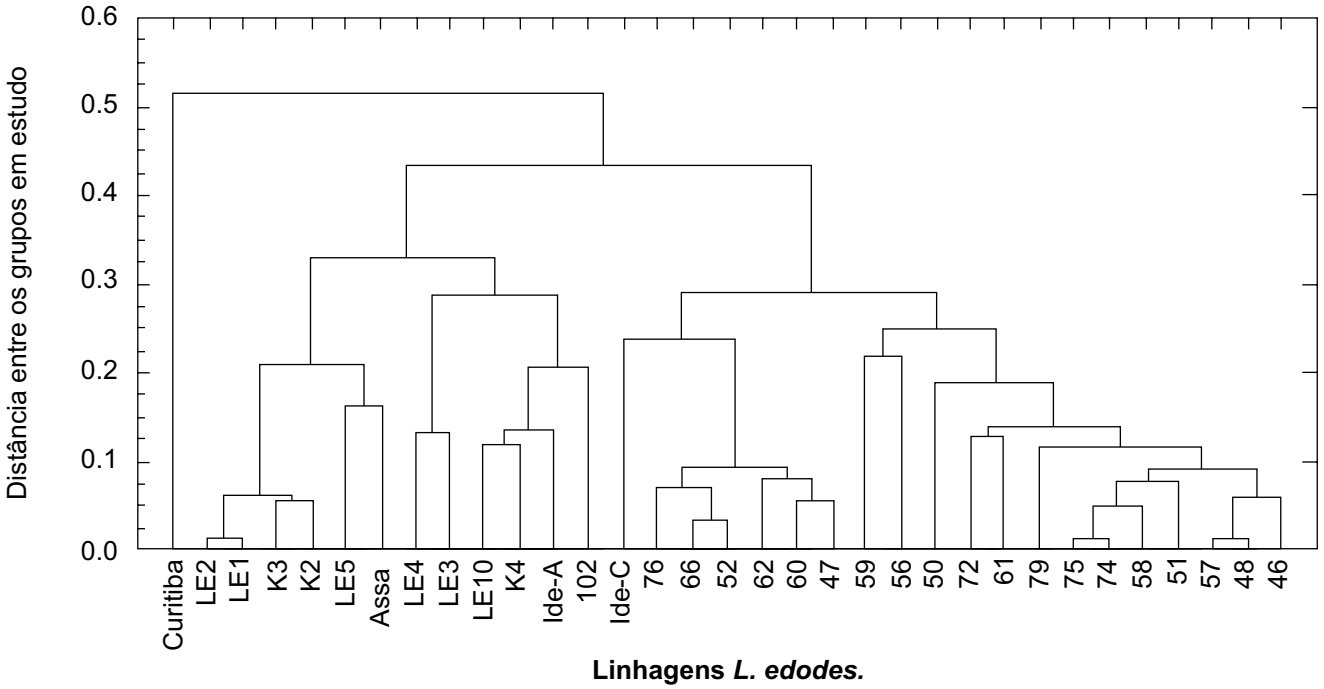

Figure 6. Dendogram from the cluster analysis and genetic distances among 34 *L. edodes* strains for polymorphism generated by the use of RAPD molecular markers.

is different from the study by Sunagawa (11) who found a large number of polymorphic bands with the OPA16 primer in a RAPD molecular marker analysis. It is suggested that the use of different strains in the two studies may be the cause of the

RESUMO

Variabilidade genética em *Lentinula edodes* analisada por respostas de desenvolvimento do micélio a diferentes condições abióticas e por marcadores moleculares RAPD

Analisou-se o comportamento de 34 linhagens de *Lentinula edodes*, quando submetidas a diferentes condições de cultivo micelial (pH e temperatura), e a variabilidade das linhagens através de marcadores moleculares RAPD. A caracterização das linhagens foi realizada observando-se o comportamento de cada uma delas quando submetidas a três pHs (5, 6 e 7) e quatro temperaturas diferentes (16, 25, 28 e 37ºC), em função da taxa de desenvolvimento e da retenção de água pelo micélio *in vitro*. O cultivo micelial foi bem sucedido em todos os pHs utilizados. Já para o fator temperatura, foi estabelecido como faixas ideais para cultivo micelial, aquelas compreendidas entre 25 e 28ºC. O conteúdo hídrico foi menor nas linhagens cultivadas a 37ºC. O padrão de RAPD foi determinado e foram testados 20 *primers* OPA (*Operon Technologies*, Inc.), dos quais 17 forneceram boa fonte de polimorfismo (OPA01 a OPA05, OPA07 a OPA14, OPA17 a OPA20). O agrupamento das linhagens baseado nos coeficientes de similaridade permitiu a separação de dois grupos de procedências geográficas distintas.

Palavras-chave: cogumelos comestíveis, RAPD, crescimento micelial

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