

DIGITAL MONITORING OF MYCELIUM GROWTH KINETICS AND VIGOR OF SHIITAKE (*LENTINULA EDODES* (BERK.) PEGLER) ON AGAR MEDIUM

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ABSTRACT

The mycelium growth kinetics and vigor of shiitake (*Lentinula edodes* (Berk.) Pegler) strains LE 96/17, LE 98/51, LE 98/53, and LE 98/56 were studied under different agar medium compositions. The strains were from the mycological collection of the Módulo de Cogumelos, Faculdade de Ciências Agrônômicas, Unesp-Botucatu, Brazil. Mycelium fragments from stock cultures were transferred to Petri dishes with Sawdust extract-Dextrose-Agar medium. The area of growth and vigor (density) of the mycelia were daily recorded with a digital camera, during incubation, until the complete colonization of the Petri dish. The images were analyzed by the freeware UTHSCSA ImageTool, v. 2.0, developed by the University of Texas Health Science Center, San Antonio. The kinetics of mycelium growth, as measured by the mycelium area (mm²), has as a deterministic component an exponential function of Gompertz. The vigor, as evaluated by mycelium color in gray scale, was similar for all strains, reached a maximal value between the 4th and 5th day of incubation and decreased further on. The velocity of growth of *L. edodes* strains was lower in enriched culture media, while vigor was higher. Digital monitoring permits a objective evaluation of the growth kinetics of *L. edodes in vitro*.

Key words: *Lentinula edodes*, shiitake, substrate, mycelium growth, strains.

INTRODUCTION

Filamentous fungi growth has been estimated by measuring the radial growth of hyphae in solid culture media over time. Growth rates vary under different environmental conditions, thus there is a pursuit for the most suitable conditions for filamentous fungal growth (4). In experimental conditions, solid culture media (agar) may be considered adequate because in the wild, fungi usually benefit from solid material such as wood substrates, animal or vegetable tissue remnants, or soil (5). Mycelial growth on semisolid media can be quantified by area, volume (5,13) or average radius measurements (3).

The microbial biomass growth over a period of time results in a typical sigmoidal curve, which may be divided into several phases with characteristic physiological properties. The first phase is named lag-phase and is characterized by smaller growth rates; the second phase is the exponential, characterized by a constant and maximal growth rate; the third phase depicts the

decline of growth rates and is sometimes regarded as a stationary phase of short duration, which is followed by the fourth and last phase, that of death.

The duration of the lag-phase depends on the nature of the inoculum and the nutrients available in culture media. The exponential phase is strongly affected by nutritional supplements as nitrogen and carbon sources. The declining growth rate phase occurs due to the exhaustion of some rate-limiting nutrients or by the accumulation of toxic metabolites. Finally, the death phase is usually followed by autolysis (5,17). Oxygen concentration and pH influence fungal metabolic processes and, consequently, their ability to make use of nutritional substances as carbon, nitrogen, vitamins, and minerals (4,6) state that the best mycelial growth, as measured by the diameter of *L. edodes* mycelium in culture media, occurred under pH 5. The accumulation of toxic final products and the production of secondary metabolites also influence growth (11).

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Nevertheless, we find it has been difficult to analyze microbial biomass by vigor of growth, as a consequence of vertical growth and the density of hyphae. We have previously attempted to assess this subjectively by applying classes to the radial growth multiplied by vigor (8,14). In the present study we sought to study the interaction between *Lentinula edodes* (Berk.) Pegler (10) strains and culture media using digital monitoring by determining the kinetics and vigor of mycelium growth by a precise and repetitive manner.

MATERIALS AND METHODS

The experiments were carried out in the laboratories of the *Módulo de Cogumelos* of the *Departamento de Produção Vegetal* of the *Faculdade de Ciências Agrônômicas*, Unesp, Campus de Botucatu, Area of Biotechnology and Agricultural Microbiology, Botucatu, SP, Brazil (Latitude 22°51' South, Longitude 48°27' West and altitude = 786 m).

Origin of isolates

The *L. edodes* strain LE 96/17, according to the producer was from Israel and cultivated on logs. The strains LE 98/51 (originated from U.S.A., according to the producer), LE 98/53 (according to the producer, originated from Japan), and LE 98/56 (isolated in Taiwan by Mr. Yeh Wen Sheng) were used for axenic cultivation, being all derived from the mycological collection of the *Faculdade de Ciências Agrônômicas, Universidade Estadual Paulista, Brazil*. These lineages present different genotypes, as revealed by RAPD (random amplification of polymorphic DNA) (9).

Culture Media

The composition (dry matter) of culture media SDA-20, SDA-40, SBDA-20, and SBDA-40 was respectively: 156 g of sawdust (SW), 20 g of wheat bran (WB), 20 g of rice bran (RB), and 4 g of calcium carbonate (CC); 116 g SW, 40 g WB, 40 g RB, and 4 g CC; 116 g SW, 40 g of sugar cane bagasse (SC), 20 g WB, 20 g RB, and 4 g CC; and 86 g SW, 30 g SC, 40 g WB, 40 g RB, and 4 g CC. All substrates were prepared in 1 L of boiling water during 15 min, filtered in cotton, supplemented with 10 gL⁻¹ of dextrose and 15gL⁻¹ of agar, filled to 1 L with dH₂O, if necessary, and finally autoclaved at 1 Kgf/cm² (121°C) for 30 min.

Shiitake strain inoculation

The culture media in Duran glass vials (1000 mL) was poured on Petri dishes under laminar flow and after complete solidification, the four strains were inoculated in all culture media, in order to enhance fungus adaptation to nutrients and diminish the interference of unknown factors during data collection. As soon as the mycelium reached the approximate distance of 10 mm from the dish's perimeter, it was transferred to a new dish with exactly the same media, under laminar flow, in order to conduct the measurements of mycelium growth. For mycelia

transferring we used a circular cutter (5-mm diameter) to cut colonized media, at the colony border, and with the aid of a small sterilized platinum handle, it was lifted and deposited in the center of the new dish, with the mycelium faced to the culture media. The dishes were then inverted and maintained in plastic bags (in order to avoid contaminations during handling) in B.O.D. chambers, at 25°C, with no photoperiod.

Monitoring of mycelium growth and vigor of shiitake

Daily photographs recorded the growth of *L. edodes* strains during incubation until the complete colonization of the Petri dish. The first photograph was taken at the same time of inoculation, but on the next day, establishing, therefore, 24-h cycles. A digital camera attached to a pedestal with an opaque dark background was installed inside the laminar flow. Thus, the position of the camera in relation to the focused platform, whereon the dishes were accommodated, was at a fixed position. The intensity of the fluorescent light reaching and reflecting from the dishes was 550 lux and 50 lux, respectively, as measured by a luximeter (2). Images were photographed with an *Agfa* digital camera (model ePhoto1280) with the resolution 307S (640 x 480 pixels, standard compression), minimal zoom, normal preview, and macro automatic focus. All other settings were standard. The images were analyzed with the freeware UTHSCSA ImageTool, v. 2.0, 1997 (*University of Texas Health Science Center, San Antonio, Texas, USA*).

Determination of the area of micelial growth

The files containing the photographs were opened in a personal computer with *Agfa PhotoWise* (camera accessory); in each photograph, the colony was copied as a quadrilateral image and then image-analyzed in the *ImageTool*. Image analysis consisted on the identification of objects that could be geometrically delimited. The objects should be comprised of a continuous region with an equal tonality. Images were analyzed in gray scale with levels that could vary from 0 (black) to 255 (white). Thresholding was performed manually for each image. Seven repeats were performed for each treatment. Images were analyzed separately for area (mm²) and vigor, depicted by the mean gray level. The mean and standard deviations of both measures were stored in a Microsoft Excel spreadsheet.

Statistical methods

It was conducted completely randomized factorial experiments (four strains and four culture media), with seven replications per treatment. Nonlinear regressions models were used to fit the mycelium growth kinetics of the *L. edodes*. After the selection procedure, the best model was choice using the residual sum of squares. The Nonparametric analysis of variance was carry out (Kruskal-Wallis test) to compare the instantaneous velocity growth (taking the first derivate of the deterministic component of the model) between treatments, by the Student-Newman-Keuls test. The procedure used as follows:

if there is (no) statistical difference between the parameters of two treatments, then we (don't) have the statistical difference between the instantaneous velocity growth.

RESULTS AND DISCUSSION

The model that best explained the growth kinetics of *L. edodes* strains in solid culture media has, in its deterministic component, the exponential of a Gompertz function (12):

$Y_{ij} = \exp \{ \alpha_j \exp [- \exp (\beta_j - \gamma_j X_{ij})] \} + e_{ij}$ where Y_{ij} is the area of growth (mm²); X_{ij} is the time in days; α , β , and γ

are the parameters model; e_{ij} = random component; the index i and j are related to the observations and treatments, respectively.

Filamentous fungi growth was determined by the α , β , and γ parameters, in which interacted during the colonization of the culture media, unleashing distinct behaviors among strains as regards culture media, as was observed by Regina (13) while studying their growth kinetics in culture media made of sawdust and sugar-cane bagasse supplemented with bran in various proportions. Growth curves of *L. edodes* mycelium in different culture media are depicted in Fig. 1.

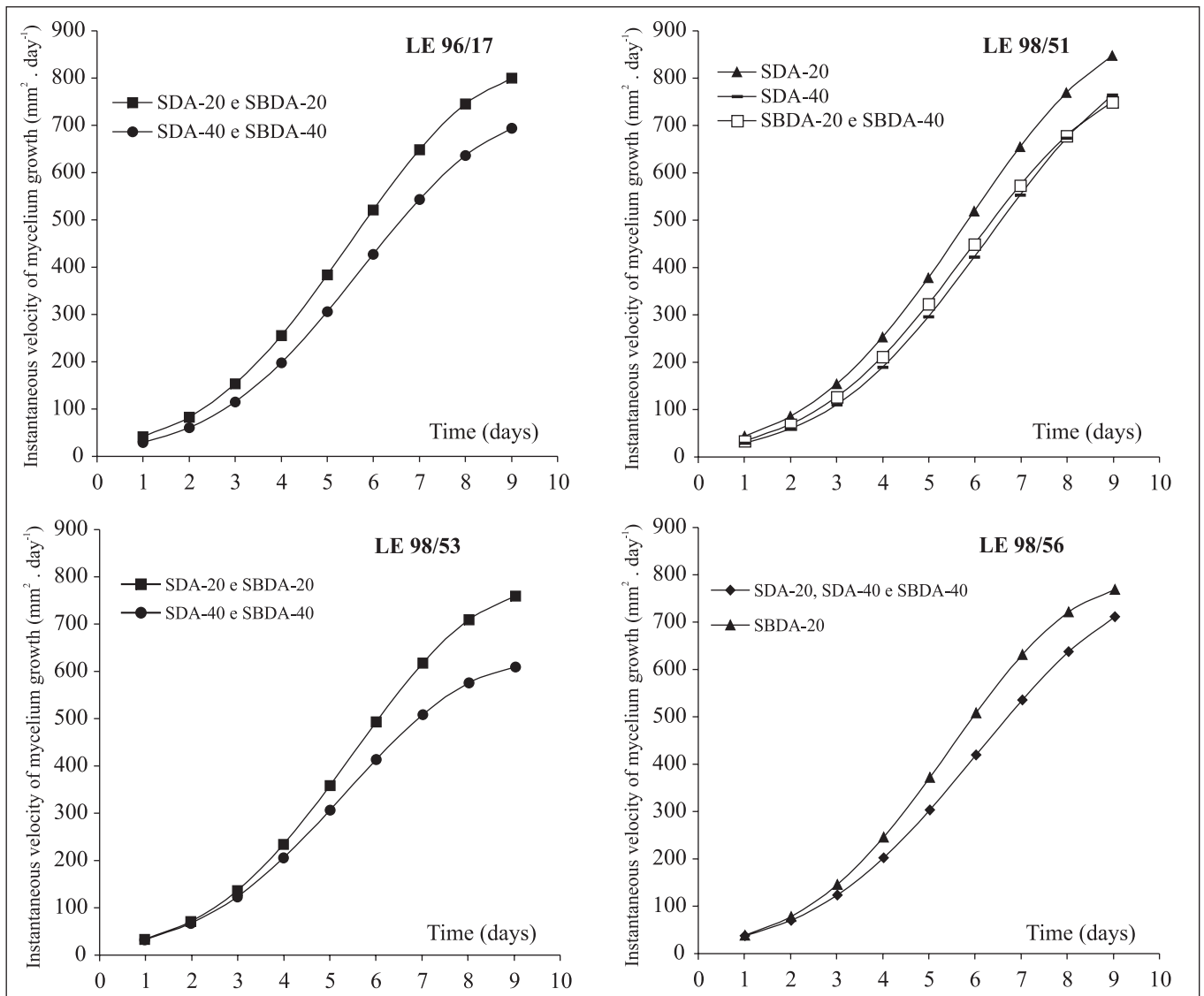


Figure 1. Instantaneous velocity growth of *L. edodes* mycelium strains LE 96/17, 98/51, 98/53, and 98/56 in the culture media SDA-20, SDA-40, SBDA-20, and SBDA-40 (radial growth in Petri dishes). In all curves plotted there are statistical difference between the instantaneous velocity growth the parameters of the treatments.

When the instantaneous velocity curves of mycelium growth of all strains are compared in function of culture media (Fig. 1), we observed that LE 98/51 presented higher instantaneous velocities in the SDA-20 media along time, followed by the strain LE 96/17 in SDA-20 and SBDA-20 media, which did not show significant differences between them, as depicted in Fig. 1. In the other hand, strains LE 96/17 and LE 98/53 presented, in comparison, inferior instantaneous velocities in the SDA-40 and SBDA-40 media. Variations in growth speed as a function of strain have been described by Boyle (1), while studying *L. edodes* growth-limiting factors.

Similarly, strain effects were also reported by others authors (15,20) studying the interaction of *L. edodes* strains with culture media constituents. The same was observed for biomass production in liquid media (19). Inter-strain metabolic differences can cause significant differences in the capability of nitrogen utilization (7). Nitrogen is a limiting factor for the mycelium growth of *L. edodes* (1). However, there is a maximal threshold, after what a decrease in growth occurs (16).

The terminology “vigor” is herein being proposed and quantified digitally, although it has already been used by other authors (8,14,18). Vigor has albeit been measured by the

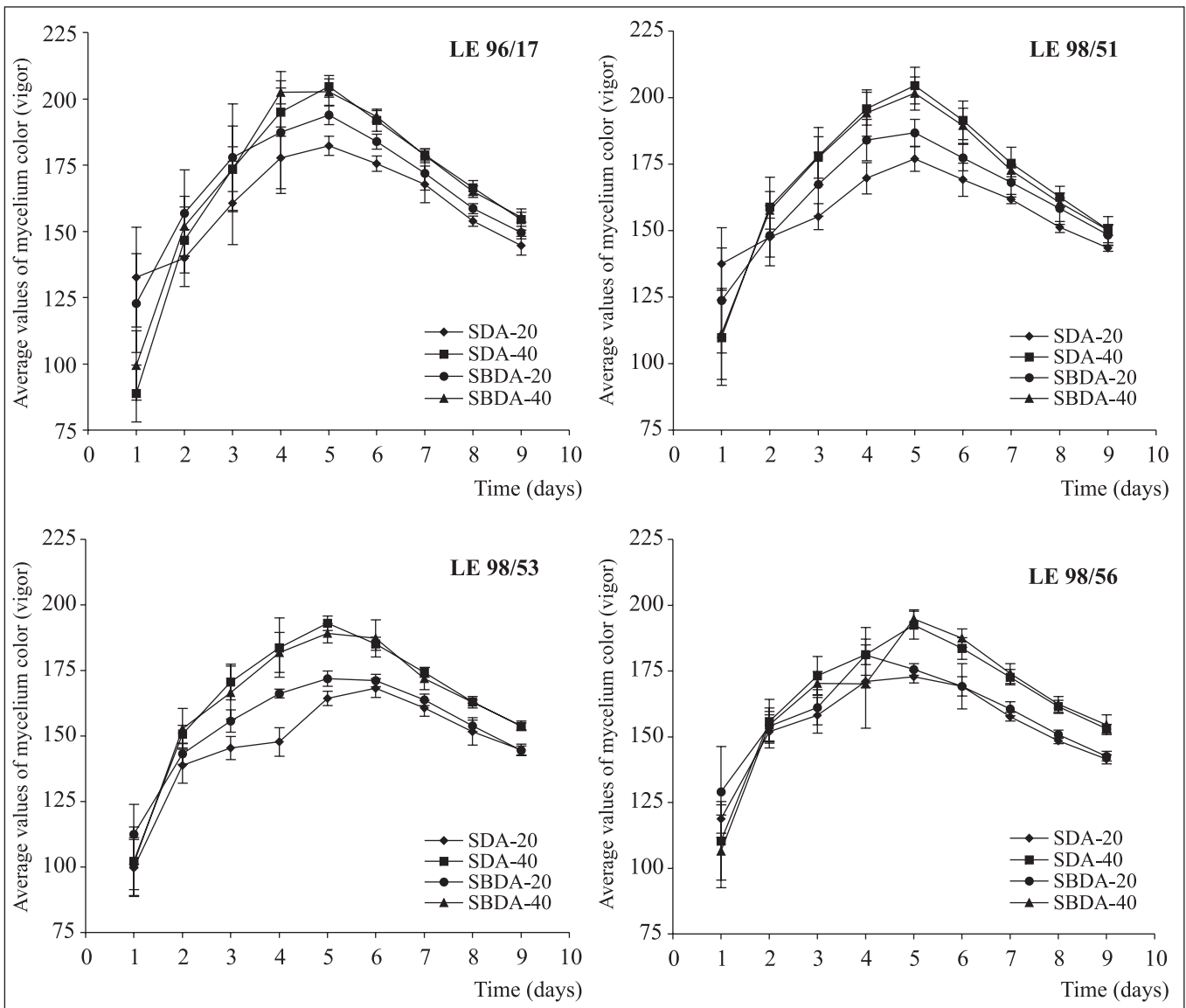


Figure 2. Average values of mycelium color in gray scale (vigor) during mycelium growth of strains LE 96/17, 98/51, 98/53, and 98/56 in function of culture media.

subjective criteria of scores. Thus, we propose to express vigor as a continuous variable obtained by digitalized images based on the mycelial density (reflecting directly the biomass) and mycelium age. Mycelium age can be expressed in function of tonality changes along time, which frequently escape the naked eye but are detectable by digital analysis.

The behavior of vigor was similar for all strains (Fig. 2). In the first day, vigor was smaller, probably due to the brownish area present on the back of the disc, used to inoculate the culture media with *L. edodes*. Starting from the 2nd day, we observed a progressive increase in the mean values, peaking between 4th and 5th days. Vigor varied as a function of the applied culture media, with exception of strains LE 98/53 in media SDA-20, wherein the maximal value occurred at 6th day. After they reached maximal vigor, we registered a decline until the last day. 95% confidence intervals evinced a decrease in amplitude of measures beginning from the day of maximal vigor. The results allowed us to verify how strains develop they increase in vigor. However, vigor decreases after 5th day, probably due to the ageing of the mycelium in the colony center, a fact that shifts the metabolism of the fungus, yielding vacuoles (17) and exudates (5). Such facts may thus contribute to the tonality changes of the mycelium, which are not detectable by naked eye.

Digital analysis permits the non-subjective evaluation of vigor of growth of strains. Apart from the evaluation on a Petri dish, digital analysis could also be used for the assessment of growth in sawdust, which has been associated with good fruiting of *L. edodes* (18).

CONCLUSIONS

The kinetics of mycelium growth, as measured by the mycelium area, regardless of strain and substrate, has as a deterministic component an exponential function of Gompertz.

The vigor of different *L. edodes* strains determined *in vitro* by digitalized image decreases from the fifth day of incubation, independently of strain and culture media. The velocity of growth of *L. edodes* strains was lower in enriched culture media, while vigor was higher.

Digital monitoring permits a objective evaluation of the growth kinetics of *L. edodes in vitro*.

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RESUMO

Monitoramento digital do crescimento e vigor do shiitake (*Lentinula edodes* (Berk.) Pegler) em meio de cultura

Avaliou-se o crescimento e vigor das linhagens LE 96/17, LE 98/51, LE 98/53 e LE 98/56 de *Lentinula edodes* (Berk) Pegler em diferentes composições de meio de cultura. As linhagens foram provenientes da Micoteca do Módulo de Cogumelos da Faculdade de Ciências Agrônômicas, Unesp, Campus de Botucatu. Os isolados foram obtidos por propagação vegetativa, pela transferência asséptica do micélio para o meio de cultura de extrato de serragem-dextrose-ágar. O crescimento e vigor do micélio foi fotografado diariamente com uma câmera digital, durante a incubação, até a colonização total da placa de Petri. As imagens foram analisadas pelo programa UTHSCSA *ImageTool* (freeware), versão 2.0, desenvolvido pela *University of Texas Health Science Center*, San Antonio, Texas. O modelo estatístico que melhor explicou a cinética de crescimento miceliano em área (mm²), das linhagens de cogumelos *L. edodes*, tem como componente determinístico a exponencial de uma função Gompertz. O vigor, avaliado através da cor do micélio (em escala de tons do cinza), revelou um comportamento similar entre as linhagens, com valor máximo entre o 4^o e 5^o dia e, em seguida apresentou declínio. Em meios de cultura mais enriquecidos, a velocidade de crescimento das linhagens de *L. edodes* foi menor e o vigor maior. O monitoramento digital permite uma avaliação objetiva do crescimento e vigor do *L. edodes in vitro*.

Palavras-chave: *Lentinula edodes*, shiitake, substratos, crescimento miceliano, linhagens

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