Article

Logistic model of the dynamics of the mycelial growth of the fungus 'Totolcozcatl' in culture media

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Abstract

Edible wild mushrooms are considered a non-timber forest resource, of nutritional, ecological, cultural and economic importance for rural communities. In the mountain mesophilic forest, in Xaltepuxtla, Puebla, the looting and overexploitation of the *Entoloma abortivum* species, known as 'Totolcozcatl', place it as a threatened species. Therefore, the objective of this work was to evaluate its dynamics of average mycelial growth, in culture media using a logistic model, for its induction process. The culture media evaluated in 2018 were: malt extract-yeast extract-polypeptone-agar (MYPA); meat peptone-dextrose-yeast extract-bacteriological agar (CYM) and malt-bran extract (EMS). Statistical analysis indicated that the MYPA and EMS substrate were statistically different from CYM ($p< 0.05$), in mycelial growth after 16 days of culture. The logistic model adjusted to the experimental data obtained in each of the substrates yielded R^2 coefficients of determination of 0.99. The model showed that the maximum predicted growth was observed in the EMS substrate (95.94 mm) and the minimum in CYM (83.23 mm). The point of maximum growth and excessive production of hyphae added to the mycelium was obtained in the EMS substrate (47.97 and 9.59 mm), followed by MYPA (45.76 and 8.83 mm) and CYM (41.61 and 6.86 mm). Finally, it is concluded that the main important and viable alternative to the induction process of the Totolcozcatl fungus is the EMS medium.

Keywords: *Entoloma abortivum* (Berk. & Curtis) Donk., culture media, growth rate.

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Introduction

Wild mushrooms are functional and essential components of forest ecosystems and provide valuable products directly to humans. Due to its gastronomic and nutraceutical properties, its demand has been increased to the detriment of its distribution and abundance in natural ecosystems even reaching its extinction (Pilz *et al*., 1996; Alvarado-Castillo *et al.*, 2015).

According to Sánchez and Mata (2012), the cultivation of edible wild mushrooms has been gaining momentum worldwide since the middle of the last century and possibly continues this trend for the following reasons: population increase, increase in demand, diversification of the fungi produced, new applications of fungi in the field of food and medicine, and attractive as an economic activity. Regarding its commercialization, Contreras *et al*. (2018) indicate that the price of the edible fungus Totolcozcatl (*Entoloma abortivum*), of terrestrial habitat, symbiote and parasite, ranges from $100.00 to $150.00 per kg, due to its scarcity in the region.

Alvarado *et al*. (2012) mention that there are two options for the rational use of this resource and its conservation: 1) the proper management and management of natural populations; and 2) the development of techniques for domestication and commercial production. Edible wild mushrooms can be cultivated in controlled environments, since being independent of other living beings, it is enough to develop a specific lignocellulosic substrate and control the appropriate temperature, ventilation, humidity and light conditions to make them grow and fructify.

In this context, Ríos and Ruiz (1993) establish that for the propagation of edible wild mushrooms for commercial purposes, it is necessary to identify the prevailing species in the region and determine the factors that influence their development and growth. Once this has been achieved, the phases prior to its propagation are obtaining mycelium, making inoculum, preparing the substrate, inoculating the substrate, incubation and fruiting.

The obtaining of mycelium is one of the determining phases of success for the propagation of the fungus, which depends largely on the culture medium providing the necessary nutrients for the development of the mycelium over time and the way in which it demands it. Therefore, the objective of this research was to evaluate the dynamics of growth of the average mycelial diameter of the fungus 'Totolcozcatl' in three culture media: malt extract-yeast extract-polypeptone-agar (MYPA), meat-dextrose peptone-bacteriological yeast extract (CYM); and malt-bran extract (EMS) and assess using a logistic model, which of the means provides the most favorable conditions for the development of the mycelium as a determining stage prior to its spread.

Materials and methods

Location of area of study

Issues of the species *Entoloma* sp. were collected in mountain mesophilic forest relics located in the Ocotitla estate, in the community of Xaltepuxtla, in the state of Puebla, between the coordinates 20° 11' 23.06" at 20° 10' 57.124" north latitude and 97° 58' 5.303" at 97° 57' 30.836" west

longitude, at an average height of 1 280 meters above sea level (Mateo-Guzmán, 2018). The climate corresponds to a semi-warm humid with rains throughout the year, precipitation of the driest month greater than 40 mm and less than 18% of winter rain with respect to the total rain and its annual average temperature ranges between 18° and 24 ° C.

Collection and disinfection of the material

Full body fungi were collected during their emergency period, which occurs between December and January, when organic substrate conditions, temperature and humidity are favorable for growth (Mateo, 2018). They were covered with foil and transported in a cooler to have fresh material for insulation. In the laboratory, this process consisted of selecting whole and healthy fungi; disinfect the surface of the fungi by means of a cotton soaked in a solution of water with detergent, then with a 10% chlorine solution and finally in a 0.2% solution of veterinary gentamicin antibiotic.

Getting mycelium

The mycelium was obtained in the three-culture media using the methodology of Ardon (2007) and Silva *et al*. (2010), which consists of isolation by context, purification process and test of mycelium growth in culture media. This last stage consisted of: a) reseeding the strains obtained from isolation by context (vegetative) in three culture media (malt extract-yeast extract-polypeptone-agar (MYPA); meat peptone-dextrose-yeast extract- bacteriological agar (CYM) and malt-bran extract (EMS); b) compare its growth in each; c) define the medium in which the mycelium best adapts and grows; d) inoculate more than five boxes by means; e) choose five of these with better appearance and absence of contaminants; f) extract a fraction of the strain in a circular form of approximately 0.5 cm; g) place it in the center of a Petri dish; h) seal it with parafilm and label it with the name of the medium and the date of inoculation; i) place the sealed and labeled box at room temperature; and j) measure the diameter of mycelial growth every two days.

Analysis of data

For the analysis of mycelial diameter growth data in the three culture media: MYPA, CYM and EMS, a one-way analysis of variance (Anova) (1V) was applied for repeated measurements (MR), Anova-1V-MR (Davis, 2002), to determine if these have any effect on the growth of the mycelium diameter of the Totolcozcatl fungus, where the culture media correspond to the categorical factor with three levels. The method consisted of: a) checking the assumptions of homogeneity between the variances of the differences between all the possible pairs of culture medium and the normality of the errors by means of the Mauchly and Shapiro-Wilk sphericity test, respectively; b) apply the Greenhouse and Geisser epsilon test or the multivariate approach, in cases where the sphericity assumption is not met; and c) apply the statistical model $y_{ij} = \mu + \pi_i + \tau_j + \varepsilon_{ij}$.

For each $i = 1, \cdots, n$ and $j = 1, \cdots, J$ in the equation, y_{ij} is the response of the growth of the mycelial diameter of the subject i at the j-th level of the culture factor or medium j, μ is the general mean of the response variable, π_i is the random effect associated with the subject which

is constant in all repetitions, τ_j is the fixed effect at the j-th level of the culture medium, and ε_{ij} is the experimental error associated with the subject i under the treatment j. It is assumed that random effects π_i are normal $N(0, \sigma_\pi^2)$ independent, random errors ε_{ij} are normal $N(0, \sigma_\varepsilon^2)$ independent and random effects π_i and random errors ε_{ij} are independent. It is assumed that the fixed effects τ_j satisfy the restriction that their sum is equal to 0. $\sum_{j=}^{t} \tau_j = 0$. This model was applied to compare the means of the variables; that is, evaluate their differences and test the following hypotheses.

H_o: The effect of the MYPA, CYM and EMS culture media is the same in the growth of the mycelial diameter *versus* H_1: at least one is different.

The hypothesis tests were performed with a level of significance $\alpha = 0.05$ and the comparison between means using the Bonferroni method (Morell, 2014) with the SPSS software (IBM Corp. Released, 2012).

Determination of the dynamics of mycelial growth

The dynamics of the growth of the fungal diameter or increase of mycelial biomass is a function of the fermentation parameters and even in the face of stress situations (Deacon, 2006; Huang *et al*., 2010). Several models have been applied to describe the kinetics of mycelial diameter growth in different types of crops, such as the linear, exponential, logistic model, as well as modifications of these (Viniegra-González *et al*., 1993; Mitchell, Von Meien *et al*., 2004). The differential equation proposed by Sarikaya and Ladisch (1997) was used to determine the dynamics of the growth of the average mycelial diameter.

$$\frac{dD}{dt} = \mu_1 D - k_1 D^2$$

Where: D= diameter of mycelial growth (mm); t= time (days), μ_1= the specific growth rate ($days^{-1}$), K= is the maximum diameter of mycelial growth (mm) and $k_1 = \frac{\mu_1}{K}$ is the growth retention factor. When the growth of D is small, the fact $k_1 D^2$ is insignificant, so the microbial population has exponential growth. However, if the surface coverage by microbial biomass is large, the mycelium growth rate will be reduced as a result of competition from a growing population for a limited substrate; that is, the effect of competition would be related only to the length of hyphae.

The model of mycelial growth dynamics, obtained from the integration of $\frac{dD}{dt}$, adjusted to the data was: $D(t) = \frac{K}{1+Ce^{-\mu_1 t}}$ where $C = \frac{c}{k_1}$, and c= is the constant of integration. The point at which the population D(t) grows rapidly from one time to another was obtained at the moment when D'(t) is maximum and D"(t)= 0.

$$\frac{d^2D}{dt^2} = \frac{d(\mu_1 D - k_1 D^2)}{dt} = 0 \text{ such that } D(t)_{máx} = \frac{K}{2}$$

And this happened when t is equal to: $\frac{K}{2}=\frac{K}{1+Ce^{-\mu_1 t}}$ such that $t_{máx}=\frac{\ln(C)}{\mu_1}$. Finally, the surplus production or number of individuals added to the population from one time to another, was obtained by: $\frac{dD}{dt}=\mu_1\left(\frac{K}{2}\right)-k_1\left(\frac{K}{2}\right)^2$ such that $\frac{dD}{dt}_{máx}=\frac{\mu_1 K}{4}$.

The parameters K, C and μ_1 were obtained; through, the logistic model adjustment to the experimental data by means of the Marquardt algorithm with the Curve Expert software version 1.3 (Hyams, 2003).

Results and discussion

Determination of mycelial diameter growth

Table 1 shows the results obtained from the average growth of the mycelial diameter of the Totolcozcatl fungus in the three-culture media. The average net growth of the mycelium was 88.5, 78.3 and 89.5 mm, for the culture medium MYPA, CYM and EMS, respectively. Differentiating between the culture media, in EMS a maximum average growth diameter of 90 mm was reached at 16 days, followed by MYPA and CYM with 89 and 78.8 mm, respectively, in the same period of time.

Table 1. Growth of the average diameter of the mycelium of the Totolcozcatl fungus, in the three culture media, expressed in (mm).

Culture medium	Measurement days								
	0	2	4	6	8	10	12	14	16
MYPA	0.05	8.5	20.8	35	50.4	64.8	77	85.3	89
CYM	0.05	7.4	15.3	27.4	38.9	51.9	59.9	71.1	78.8
EMS	0.05	9.3	23	36.2	50.6	66	88.3	90	90

Based on the variability observed during the development of the mycelium and the adjusted data, Figure 1 shows that the development in average mycelial diameter of the Totolcozcatl fungus was more favorable in the EMS culture medium, managing to colonize the Petri dish over a period of 14 days, followed by MYPA and CYM.

Variance analysis of repeated one-way averages (Anova-MR-1V) for culture media

In order to determine if the culture media have any effect on the average growth of the mycelial diameter of the Totolcozcatl fungus, the statistical technique of the Anova-MR-1V was applied to the experimental data. The culture medium corresponded to the categorical factor with three levels, that is, the culture media: 1-MYPA, 2-CYM and 3-EMS, respectively. The growth of the average diameter of the mycelium was defined as the dependent variable; that is, petri dish 1. Effect of MYPA, petri dish 2. Effect of CYM and petri dish 3. Effect of EMS. The sample size was eight, which are times the data were taken.

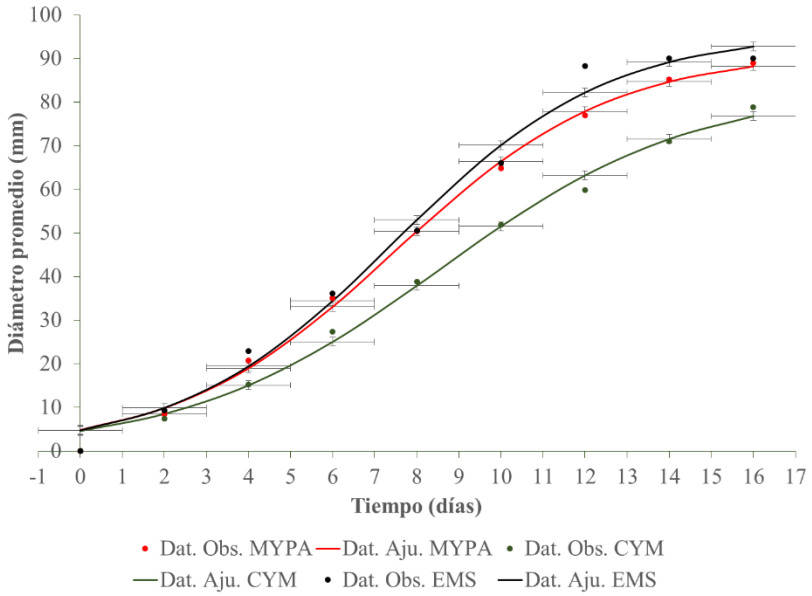

Figure 1. Development of the average mycelial diameter of Totolcozcatl fungus strains, grown in three culture media. MYPA= malt extract-yeast extract-polypeptone-agar; CYM= meat peptone-dextrose-yeast extract-bacteriological agar; and EMS= malt-bran extract.

In Table 2, the results of the Mauchly sphericity test indicate that the variances of the differences between each two levels of the factor are not equal. This means that the matrix of variances and covariances is not circular or spherical, since the critical level associated with the statistic (significance: 0.014) is not greater than 0.05. Consequently, the univariate F statistic was used, applying a corrective index called epsilon, which expresses the degree to which the variance-covariance matrix moves away from sphericity. Under conditions of perfect sphericity, epsilon must be worth 1. Epsilon was estimated by the Greenhouse-Geisser and Huynh-Feldt statistic, being the first of them more conservative. Epsilon was also estimated with the lower limit value, which expresses the value it would adopt in the case of extreme breach of the sphericity assumption.

Table 2. Results of the Mauchly sphericity test.

Effect within subjects	Mauchly W statistic	Chi-Square Approach	GL	*p*	Epsilon		
					Greehouse-Geisser	Huynh-Feldt	Lower limit
Treatments	0.239	8.586	2	0.014	0.568	0.604	0.5

GL= degree of freedom; *p* = significance.

Therefore, the correction of the degrees of freedom of F (both in the numerator and in the denominator) was made by multiplying them by the estimated value of epsilon. Consequently, in Table 3, the results obtained in the four F statistics indicate that the null hypothesis is rejected, since the critical level associated with each of the tests is less than 0.05, this means that there are statistically significant differences in the fungus development in at least one of the three culture media.

Table 3. Test of intra-subject effects.

Source		Sum of squares Type III	Degrees of freedom	Mean squares	F	Significance
Treatment	Assumed sphericity	727.85	2	363.92	21.06	0
	Greenhouse-Geisser	727.85	1.136	640.85	21.06	0.002
	Huynh-Feldt	727.85	1.208	602.35	21.06	0.001
	Lower limit	727.85	1	727.85	21.06	0.003
Error (treatment)	Assumed sphericity	241.972	14	17.28		
	Greenhouse-Geisser	241.972	7.95	30.44		
	Huynh-Feldt	241.972	8.458	28.61		
	Lower limit	241.972	7	34.57		

Comparison of means by means of the Bonferroni test

The results obtained from the two-to-two comparisons between the culture media according to the critical levels associated with each comparison indicate that there are only significant differences between the culture medium 2 with respect to 1 and 3, while between 1 and 3 no there are differences (Table 4). This first analysis indicated that the MYPA and EMS culture media are the most favorable for the growth of the average mycelial diameter of the Totolcozcatl fungus than the CYM.

Table 4. Comparison of means between culture media.

Treatment (I)	Treatment (J)	Mean difference (I-J)	Standard error	Significance[b]	95% confidence interval for the difference[b]	
					Lower limit	Upper limit
1	2	10.006*	1.81	0.003	4.344	15.669
	3	-2.831	1.306	0.201	-6.916	1.254
2	1	-10.006*	1.81	0.003	-15.669	-4.344
	3	-12.838*	2.825	0.008	-21.672	-4.003
3	1	2.831	1.306	0.201	-1.254	6.916
	2	12.838*	2.815	0.008	4.003	21.672

[b]= setting for multiple comparisons= Bonferroni.

The differences found in the development of diameter growth between the three means used are associated with their chemical composition. The EMS medium has nutritional characteristics that favor the development of the species, since it provides nutrients such as maltose, glucose and mainly wheat bran, which supplies cellulose, polysaccharides and hemicellulose, which, in the natural habitat of the fungus, cellulose is always present in decomposing matter (Mateo, 2018). This favors fungi to increase the branching of their hyphae and, consequently, the amount of biomass and the surface area of the mycelium (Harris, 2008). On the contrary, if nutrients are limited, the mycelium tends to be less branched (Prosser and Tough, 1991).

Determination of the dynamics of mycelial growth

The model adjusted to the growth data measured as the average mycelial diameter of the Totolcozcatl fungus, in the three culture media was the logistic one, which coincided with what was stated by Sarikaya and Ladisch (1997), who mention that the mycelial diameter measurements present a good fit with a logistic model, though, this adjustment is best when the area of the colonies is used as a variable.

According to Madigan *et al*. (2004), this characteristic behavior of microbial development, evidences three stages. The first is latency, associated with the synthesis of enzymes that allow the use of nutrients from the culture medium. The second is exponential, obtaining in this the maximum growth point $\left(D(t)_{máx}= \frac{K}{2}\right)$ and the third is stationary, where the growth rate decreases due, possibly, to the lower availability of nutrients and is where maximum growth is reached (K). In the three adjusted models, their coefficients of determination R^2 were close to 0.99 (Figure 2, 3 and 4).

Regarding the dynamics of the development of the diameter of the mycelium of the Totolcozcatl fungus in the MYPA medium, in Figure 2, the adjusted model indicates that its maximum growth, also known as load capacity, was achieved in K= 91.51 mm, in a period 16 days. While the point at which hyphae grow rapidly from one time to another was $D(t)_{máx}= \frac{K}{2}= \frac{91.51}{2}=45.76$ mm and reached at $t_{máx}= \frac{\ln(C)}{\mu_1}= \frac{\ln(17.88)}{0.39}=7.48$ days. The surplus production or the maximum number of hyphae or ramifications added to the mycelium; that is, the optimal growth of the average diameter of the mycelium was $\frac{dD}{dt}_{máx} = \frac{\mu_1 K}{4}= \frac{0.39*91.51}{4}=8.83$ mm and similarly achieved at 7.48 days and takes place in $\frac{K}{2}=45.76$.

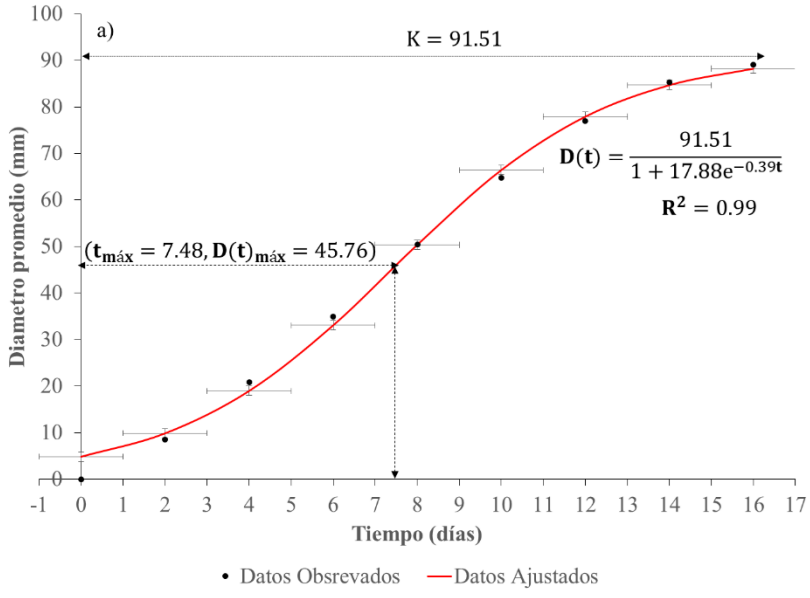

Figure 2a. Development of the average mycelial diameter of Totolcozcatl fungus strains grown in the MYPA culture medium.

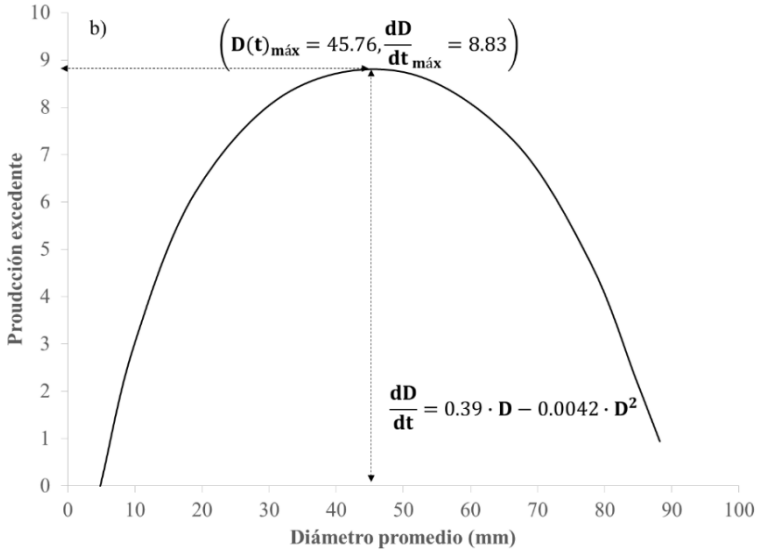

Figura 2b. Surplus production or number of hyphae added to the mycelium from one time to another, depending on the size of its population.

According to Madigan *et al*. (2004), at the beginning, the sufficiency of resources, in space and food, allows hyphae to grow in number and individually almost without restrictions, geometrically, since each unit of increased diameter means a decrease in the availability of resources. Consequently, the speed with which the hyphae was growing is gradually reduced until all the resources of the growth medium are consumed as quickly as they are produced, and the mycelium stops growing.

This point is known as carrying capacity as mentioned above (K; Figure 2a). If you consider only the number of hyphae (biomass) that is added to the mycelium from one time to another (surplus production), it increased in the early stages, reaching a maximum and began to decrease symmetrically to the initial part, reaching zero when the size of the mycelium reached the value of K ($D(t)_{máx}=\frac{K}{2}$) (Figure 2a). The greatest possible number of hyphae that could be added to the mycelium during a given period (maximum productivity or maximum net growth) is also obtained just half of K ($\frac{dD}{dt}_{máx} = \frac{\mu_1 K}{4}$) (Figure 2b).

The model adjusted to the experimental data with the CYM and EMS culture medium (Figure 3 and 4) indicate that its loading capacity and the point of maximum growth was close to (K= 83.23 mm and $D(t)_{máx}$= 41.61 mm) 9% y (K= 95.95 mm and $D(t)_{máx}$= 47.97 mm) 5% smaller and larger than that corresponding to the MYPA, respectively (Figure 3a and Figure 4a). While the excess production or number of hyphae, added to the mycelium from one time to another was $\frac{dD}{dt}_{máx} = 6.86$, for the CYM substrate, and $\frac{dD}{dt}_{máx} = 9.59$, for the EMS substrate, resulting close 22 and 9% lower and higher than the MYPA, respectively (Figure 3b and Figure 4b). Finally, the time in which the hyphae grow rapidly was $t_{máx}$= 7.48 for the CYM medium and $t_{máx}$= 7.37 for the EMS medium, resulting in about 15% and 1% greater and less, respectively, than in MYPA.

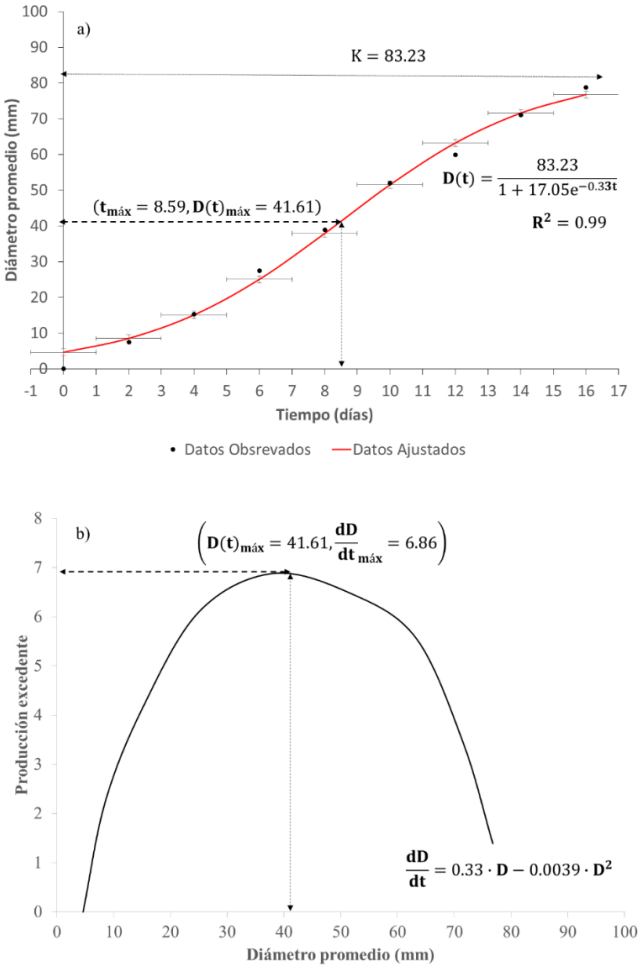

Figure 3. a) Development of the average mycelial diameter of Totolcozcatl fungus strains grown in the CYM culture medium; and b) Surplus production or number of hyphae added to the mycelium from one time to another, depending on the size of its population.

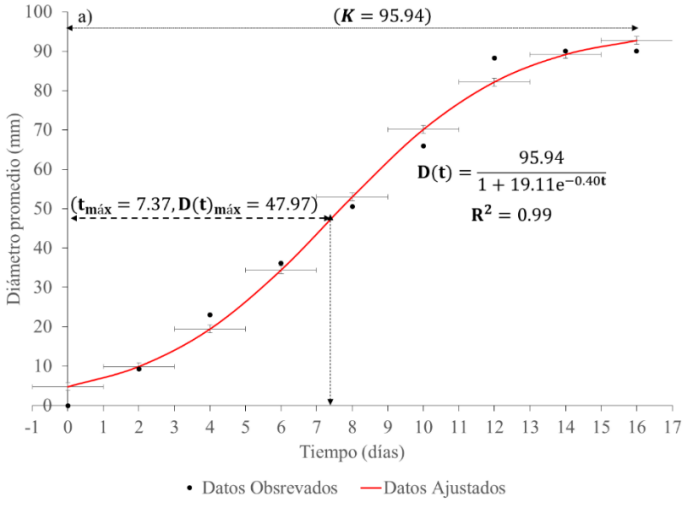

Figure 4. a) Development of the average mycelial diameter of Totolcozcatl fungus strains grown in the EMS culture medium.

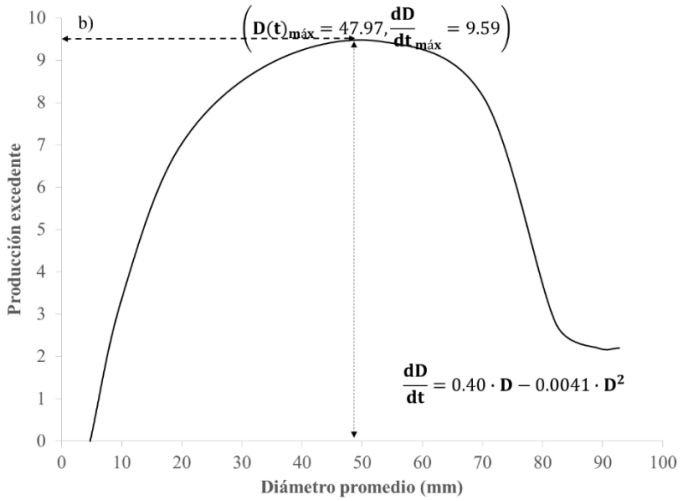

Figura 4b. Surplus production or number of hyphae added to the mycelium from one time to another, depending on the size of its population.

Conclusions

The adjusted mathematical models that explain the mycelial growth in each culture medium, in terms of goodness of fit, revealed a high percentage of the variability of the logarithms of the counts or measurements of the hyphae over time. Likewise, these functions allowed estimating: point of maximum growth (K) of the mycelium (in diameter), at which point the hyphae grow rapidly from one time to another, surplus production or number of hyphae added to the mycelium in each culture medium and less time in which it is reached. Based on these results, it is concluded that the EMS medium is the most viable medium for the Totolcozcatl fungus induction process.

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