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Components of Liquid Media on the Production of High Spore Concentrations of *Lecanicillium lecanii* (Zimm.) Gams and Zare

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Abstract: Two *Lecanicillium lecanii* isolates, ATCC26854 and V3, were evaluated for spore production in liquid media. Both isolates have interesting known properties for the production of high concentrations of chitinases (ATCC26854), and an outstanding pathogenic activity against the green cabbage aphid, *Brevicorine brassiacae* (L.). The isolates were grown on thirteen different liquid media, which had been used to produce other entomopathogenic Hyphomycetes. Experiments were carried out at 27 ± 1 °C with a 12:12 photoperiod using shake flasks. The production of spores was quantified during a seven days period, and the effects of the media were evaluated by determining spore concentration and morphology. Submerged conidia yields were higher with ATCC26854 than with V3 in all thirteen media, while higher concentrations (5.3×10^9, 4.6×10^9 and 3.4×10^9 conidia/mL) were found with ATCC26854 isolate in the Camaron, Minerales and Jenkins-Prior medium, respectively; lower yields (2.3×10^8, 2.2×10^8 and 2.3×10^8 conidia/mL) were found with the V3 isolate in Catroux, TKI and Camaron media, respectively. Spore production curves were adjusted to different sigmoid models. The process was better explained by the Richards model ($r^2 = 0.99$). Concerning conidia morphology, submerged conidia seemed to look like aerial conidia, but they were different in size (ATCC26854 2.73-6.99 μm and V3 5.28-14.29 μm); however, the dimensions fall within the ranges reported for *L. lecanii*. The analysis of shake flask cultures with the Richards model allowed selecting two low-cost liquid medium, Camarón and Jenkins-Prior, for scaling up conidia production for use in aphid biological control programs.

Key words: Conidia, *Verticillium lecanii*, liquid fermentation, spore yields.

1. Introduction

The fungus *Lecanicillium* (*Verticillium*) *lecanii* (Zimm.) Viegas is an important pathogen for a wide range of insect pests, including aphids, aleyrodids and scales [1-3], it is less frequently reported on thrips, coleopterans and orthopterous insects [4-6]. This fungus is considered as an entomopathogen with high potential as a biocontrol agent.

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The mitosporic fungus (Hyphomycetes) *L. lecanii* grows rapidly, producing mycelium or blastospores in liquid media [2, 7, 8]. Production techniques in liquid cultures can be controlled in sterile conditions, reducing production time, and allowing to scale it up relatively easy [9]. Nevertheless, blastospores, thin-walled mycelial fragments, are short lived and do not survive in adverse environments [10, 11]. Because of that, several researchers have directed their efforts to developing conidia production in submerged cultures [12-16].

Whereas nutritional studies for improving growth and sporulation in *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* [12, 15, 17-19] have been successfully developed, reports regarding the mass production of *L. lecanii* in submerged liquid medium are quite scant [20].

Mathematical functions that provide a suitable description of the sporulation dynamics of different isolates could substantiate the research on this area. According to Declerck et al. [21], statistical models can help: 1) to rank the spore production of select isolates with high sporulation and 2) to determine the impact of several factors such as nutriments, pH, etc., on sporulation dynamics.

In this study we looked into the effects of liquid medium components on the spore production of two *L. lecanii* isolates. The main objective was to determine whether *L. lecanii* is able to produce conidia in a submerged culture, and compare sporulation dynamics in different liquid cultures using statistical models.

2. Materials and Methods

2.1 Isolates and Inoculum Preparation

Two *L. lecanii* isolates were evaluated: 1) V_3, a Mexican isolate of *Diatraea saccharalis* from the Insect Pathology Laboratory, Postgraduate College, previously proved to be effective in the control of the cabbage aphid *Brevicorine brassicae* (L.) [22]; 2) ATCC26854 (ATCC) isolate, from the American Type Culture Collection (ATCC, USA) isolated from scale *Lecanium corni* (Bouché) and provided by the Biopolymers Laboratory, UAM-I (Universidad Autónoma Metropolitana-Ixtapalapa).

Both isolates were reactivated by passing them through *Brevicorynae brassicae* (Homóptera: Aphididae) nymphs; they were then reisolated and cultured on Sabouraud Dextrose Agar (SDA) plates at 24 °C for 15 d. Conidia were harvested from plates and a 0.01% suspension was made adding a sterile solution of Tween 80; this suspension was shaken vigorously in a vortex until homogeneity. Finally, the concentration of conidia was determined using a Neubauer chamber under a light microscope (400×).

2.2 Liquid Culture Medium

The experiment was established in a completely randomized design with 13 treatments and three replicates (Table 1). The 13 treatments were: 1) Adamek medium [23], originally described for the production of submerged spores of *Metarhizium anisopliae* (Metschnikof) Sorokin and successfully used for M. *flavoviride* propagation [24]; 2) Jenkins-Prior medium developed for the production of submerged conidia of *M. anisopliae* var. *acridum* (= *flavoviridae*) [14]; 3) "Agua" medium, a modification of the Jenkins-Prior medium using tap water (pH = 7.0) instead of distilled water, which also favors the presence of carbonates, calcium chloride, sodium hydroxide, iron and yeast extract [24]; 4) Catroux and Paris medium, developed for the production of high concentrations of blastospores of *Beauveria brongniartii* (Saccardo) Petch [25, 26]; 5) Jackson's medium, which was developed for the production of desiccation tolerant blastospores of *Paecilomyces fumosoroseus* (Wize) Brown and Smith [27]. This medium was slightly modified by removing corn steep liquor; 6) TKI medium [9], used for *B. bassiana* conidia production in liquid fermentation; 7) Jenkins and Prior medium [14]; 8) "Conidias" medium, which was used in the first works that reported the production of conidia non-blastospores of *M. flavoviridae* and *P. fumosoroseous* [15, 28]; 9) SDA medium, chosen as control medium; 10) "Camaron" medium (SDA supplemented with shrimp powder), developed for *L. lecanii* due to the fact that it favors the production of high concentrations of chitinolytic enzymes [29]; 11) "Pulgon" medium (SDA supplemented with aphid powder); 12) "Minerales" medium, a slightly modified Jackson's medium reported in 2003 [19], with a high yield of desiccation tolerant *P. fumosoroseus* blastospores; and 13) "Trehalosa" medium, used by Hallsworth and Magan to improve the physiological quality of conidia [30].

Table 1 Composition from thirteen liquid culture media to propagation in flask waved from two *L. lecanii* isolations.

Components g/L	Liquid culture media												
	Paris	Catroux	Jackson	Adamek	Jenkins-Prior	TKI	Agua	Conidias	Minerales	Camarón	Pulgón	Trealosa	SDA
pH	6.7	5.8	5.0	6.6	6.7	4.0	7.0	6.3	5.4				
KH_2PO_4	0.36	6.8	2			5			2				
$MgSO_4 \cdot 7H_2O$	0.6	0.1	0.3			2			0.3				
$Na_2HPO_4 \cdot 12H_2O$	1.42												
$FeSO_4 \cdot 7H_2O$			0.05						0.05				
$MnSO_4 \cdot H_2O$			0.016			0.0025			0.016				
$ZnSO_4 \cdot 7H_2O$			0.014			0.0025							
$CoCl_2 \cdot 6H_2O$			0.036										
KCl	1												
$CaCO_3$		2											
$CaCl_2$			0.4			0.05							
KNO_3		5				10							
$NaNO_3$													
NH_4NO_3	0.7							0.7					
$FeCl_3 \cdot 6H_2O$						0.012							
$Co(NO_3)_2 \cdot 6H_2O$						0.00025							
$Na_2MoO_4 \cdot 2H_2O$						0.0002							
$CuSO_4 \cdot 5H_2O$						0.0005							
Saccharose					30		30						
Dextrose	20	20	80	20		50		30	40	40	40		40
Trealose												40	
Yeast extract	5	20		20	20		30	1					
Shrimp waste										10			
Beef peptone									5	5	5	5	5
Casein hidrolizade			13.2						5	5	5	5	5
Vitamins[a]			3.15										
Aphid (*B. brassicae)*											5		

[a]Vitamin mixture which every mL contains: palmitate from vitamin A 2,500 International Unitis (IU), colecalciferol 667 IU and ascorbic acid 50 mg.

2.3 Culture Conditions

Cultures were prepared by inoculating previously sterilized medium for 15 min at 120 °C, with a suspension of aerial *L. lecanii* (ATCC26854 or V3) conidia which yielded a final concentration of 5.4 × 10^7 spores/mL. The flasks were then incubated on a Lab-line Environm-Shaker at 160 rpm and 27 ± 1 °C. Treatments in experiments consisted of 250 mL flasks containing 100 mL of each specific medium, each replicated three times at three different moments over a period of time (three months). Thus, three samples of each tested medium were taken from shaken flasks for counting spores.

2.4 Estimation of Spore Production

60 µL samples were taken from the flasks during seven days every 24 h in order to determine the concentration of conidia concentration. The concentration of spores was determined using a common Neubauer chamber. During the counts, blastospores and conidia were distinguished. Blastospores were considered hyphal bodies, monocellular, with oblong or cylindrical structure, while conidia were ovoid or cylindrical, with thin walls, and originated from phialides.

2.5 Size of Spores

Image Tool V. 3.0 analysis was used to determine spore size using a Pixera camera connected to a bright light microscope at 400× on a sample of 100 spores incubated for 10 at 25 ± 2 °C in solid medium SDA, and on a sample of 100 spores incubated for seven days at 25 ± 2 °C in "DSB" medium [31].

2.6 Statistical Analysis

2.6.1 Comparison of Culture Medium and Isolates

An experiment was established on a completely randomized design to select the culture medium with maximum yield of conidia. Sporulation data and spore size data were subjected to analyse of variance followed by a Tukey test. A Scheffé test was used to compare different components of the media, grouping them under the following criteria: carbon source (Saccharose, Dextrose, Trehalose), nitrogen source (Yeast extract, meat peptone and hydrolyzed casein), quantity of minerals (highly mineralized, mineralized, and not mineralized), pH, N and C.

2.6.2 Adjustment of Sporulation Models

Software Curve Expert, version 3.1, was used to evaluate spore yield. Three growth models were adjusted to the sporulation dynamics of *L. lecanii*:

Logistic, $y = \frac{a}{1 + be^{-cx}}$, Gompertz, $y = ae^{-e^{b-cx}}$ and Richards, $y = \frac{a}{(1 + e^{b-cx})^{1/d}}$,

where, x = time (h), y = production of accumulated spores, representing the number of accumulated spores produced when the stationary phase had reached the maximum height of the curve; b, an adjustment constant; c, growth rate; and d, curve form parameter. Correlation coefficients, variances and different parameters of the model were calculated for each culture medium. The parameters of the Richards model for both *L. lecanii* isolates were analyzed (α = 95%). The residues and the r^2 values were used to evaluate the adjustment of the models to the data (SAS v8).

3. Results

Spore production: The components of culture medium affect the production of spores in *L. lecanii* isolates (Figs. 1a and 1b). The ATCC26854 isolate produced a higher yield of submerged conidia than the V3 isolate.

3.1 Growth Curves

Both *L. lecanii* isolates followed normal sporulation dynamics (lag phase, log phase, and stationary phase). In the ATCC26854 isolate, the lag phase was observed 48 h after inoculation, while in the V3 isolate, this phase was observed after 24 h. The

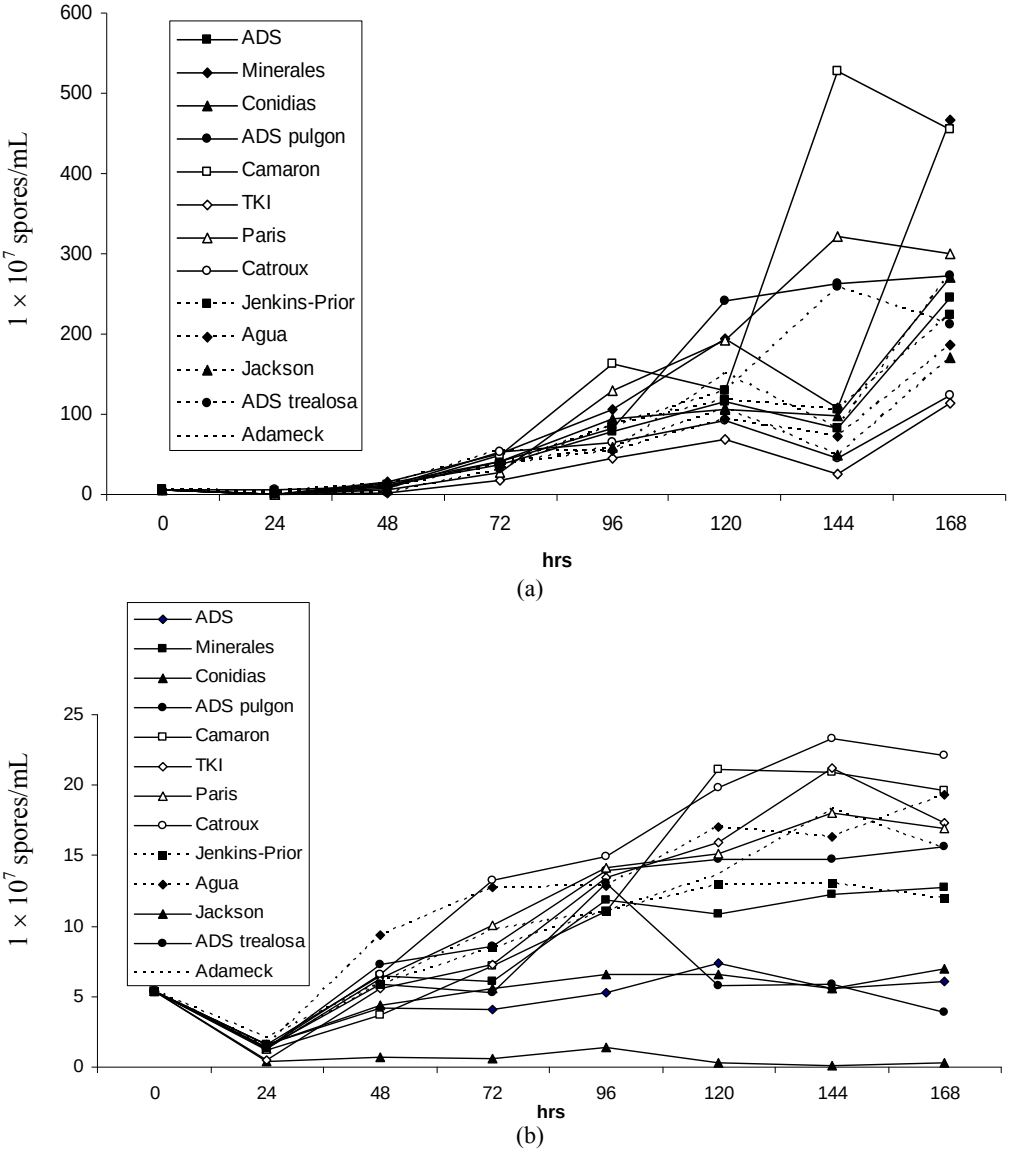

Fig. 1 Spore production after the same time periods in different liquid media for the two isolates ATCC26854 (a) and V3 (b).

log phase was different for each isolate and was affected by the composition of the culture (Figs. 1a and 1b). With ATCC26854, the spore yield in Camarón media reached > 5×10^9 spores/mL of broth at 144 h. With V3 in the same medium, spore production increased gradually from 24 h to 120 h, reaching more than 2×10^8 spores/mL at 168 h. Spore production fell back with ATCC in most liquid media. However, at 168 h it started to increase again. ATCC produced higher amounts of spores compared with V3. In most mediums, the spore yield reached > 1×10^9 spores /mL (average of 2.7×10^9 spores/mL). Among the most productive media for isolate V3, TKI, Camarón and Catroux, the spore yields reached 2×10^8 spores/mL of both isolates, (average of 1.4×10^8 spores/mL).

3.2 Effect of the Media Components

The carbon source had no significant effects on the media that favored the production of higher amounts of conidia with ATCC or V3, (P = 0.1635). Nevertheless, the amino acids source was important, as ATCC seems to prefer a media with meat peptone and hydrolyzed casein rather than media with yeast

extract (P = 0.0001) such as "Camarón" and "Minerales", which supported high conidia production (530 × 10^7 spores/mL and 460 × 10^7 spores/mL, respectively). Conidia yield with ATCC in the TKI and with Jackson's highly mineralized medium shows significant differences with respect to the less mineralized media (P = 0.0001), whereas no significant differences were observed with respect to V3 (P = 0.2502). Regarding the pH, the spore yield with ATCC does not show significant differences, whereas V3 had a slight preference to alkaline media over neutral ones (P = 0.0028).

The culture media had an important effect on the production of spores, showing that organic sources of nitrogen such as yeast extract, and inorganic sources such as potassium nitrate, ammonium nitrate, and cobalt nitrate, can, with a suitable supplementation of microelements, foster the sporulation of the fungus.

The average spore yield for V3 (Table 2) was not significantly different when using the Catroux, TKI or "Camarón" media, though the maximum spore yield in the Camarón medium was reached at 24 h, much before (120 h) than in the other two media. For ATCC (Table 3), the highest spore yields were obtained in the "Camarón" and Minerales media; in the former, maximum spore yield was reached 24 h earlier than in the latter. The highest spore production level for V3 yielded 46 times more spores than the initial inoculum after seven days of incubation, while in the best media for ATCC the yield was over 1,000 times compared to the initial inoculums, analysis of variance (ANOVA) (α = 0.05).

Spore production potential differs between both isolates. The average spore production (Scheffe's test) shows that the V3 isolate has less production potential compared with ATCC (Table 4). The V3 isolate seems to differ from ATCC with respect to its short or absent lag phase, lower spore production and large spore size.

3.3 Comparison of Spore Size in Solid and Liquid Media

Spore size, expressed in length and diameter as determined in ADS's plates after 15 days of incubation and in DS both after 168 h of incubation, are showing in Fig. 2. The length of ATCC spores in a solid medium varied from 2.00 μm to 3.54 μm (average of 2.96 ± 0.37 μm) and from 2.73 μm to 6.99

Table 2 Sigmoid growth models.

Model	Equation
Logistic	$y = \frac{a}{1 + be^{-cx}}$
Gompertz	$y = ae^{-e^{b-cx}}$
Richards	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$

Table 3 Maximum spore production for V3 isolate.

Culture media	Incubation period (h)	Average number of spores ± SD (1 × 10^7 spores/mL)				
Catroux	144	23.29 ± 1.72	a			
TKI	144	22.60 ± 0.26	a			
Camarón	120	22.57 ± 7.64	a			
Agua	168	18.82 ± 3.83	a	b		
Paris	144	17.74 ± 1.18	a	b		
Adamek	144	17.09 ± 6.17	a	b	c	
Pulgón	168	15.65 ± 1.65	a	b	c	
Minerales	168	12.58 ± 2.94		b	c	
Jenkins-Prior	144	12.34 ± 0.49		b	c	
Trealosa	96	12.02 ± 1.61		b	c	
DS	120	7.37 ± 0.19			c	d
Jackson	168	7.30 ± 1.98			c	d
Conidias	168	0.33 ± 0.21				d

Averages with different letters are significantly different (ANOVA, α = 0.05; Tukey test).

Table 4 Maximum spore production for ATCC26854 isolate.

Culture media	Incubation period (h)	Average number of spores and SD (1×10^7 spores/mL)								
Camarón	144	532.49 ± 1.72	a							
Minerales	168	469.55 ± 0.26	a							
Jenkins-Prior	168	345.31 ± 7.64		b						
Paris	144	334.15 ± 3.83		b	c					
Pulgón	168	273.6 ± 1.18			c	d				
Conidias	168	264.69 ± 6.17			c	d				
Adamek	168	262.64 ± 1.65				d				
Trealosa	144	252.29 ± 2.94				d				
DS	168	245.55 ± 0.49				d	e			
Agua	168	174.99 ± 1.61					e	f		
Jackson	168	166.16 ± 0.19						f		
Catroux	168	122.07 ± 1.98						f		
TKI	168	106.86 ± 0.21						f		

Averages with different letters are significantly different (ANOVA, $\alpha = 0.05$; Tukey test).

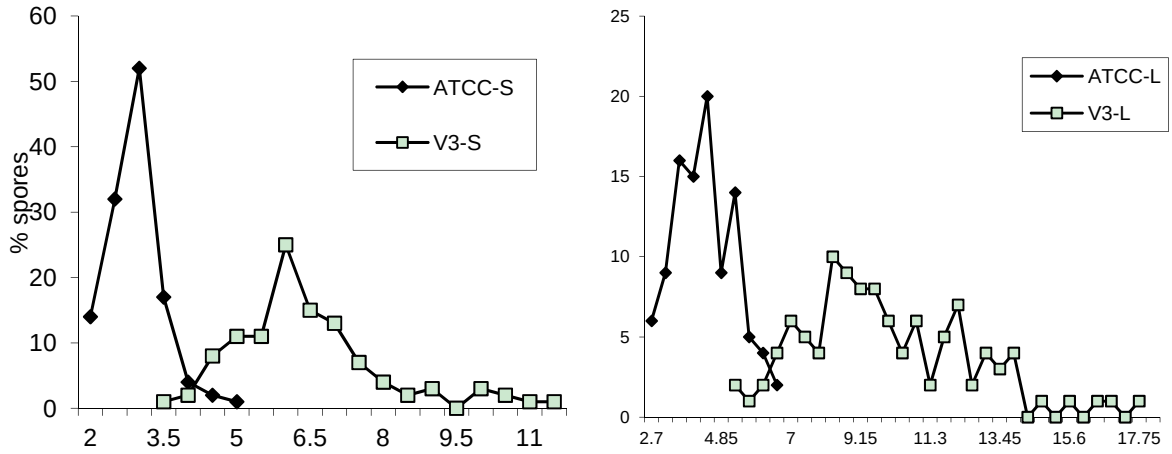

Fig. 2 Spore length distribution from *L. lecanii*in Petri dishes with ADS (a) and liquid culture, DS medium (b).

μm (average of 4.56 ± 0.94 μm) in a liquid medium, while in V3 the spore length in a solid medium ranged from 3.56 μm to 9.44 μm (average of 6.57 μm) and from 5.28 μm to 14.29 μm (average of 9.88 ± 2.2 μm) in a liquid medium. In the solid medium (Fig. 2a) the spores have more uniform sizes than in the liquid fermentation (Fig 2b), where the sizes tend to have a wide distribution, with more variability, due to the fact that the spores absorb water and swell up, causing an increase in their size. Spore size increase in liquid fermentation ranges from 50% (in V3) up to 80% (in ATCC) compared with the values obtained in a solid substrate.

The width range of *L. lecanii* spores varied. Mean spore width with ATCC in solid medium (FS) was 0.99 ± 0.26 μm, whereas in liquid fermentation (FL) it was 1.43 ± 0.25 μm. For the isolate V3, the spores from FS had a smaller width range (1.74 ± 0.43 μm) than FL spores (2.81 ± 0.7 μm).

3.4 Growth Model and Strain Comparisons

Three models, Logístic, Gompertz and Richards, had a good fit to the growth curves of *L. lecanii* (r^2 = 0.97). Nevertheless, the Richards model yielded a better fit in the lag and initial growth phases of *L. lecanii* isolates. Fig. 3 shows an example of the fit of the data set to the Richards model with respect to isolates ATCC growth in "Camarón" (A), and "Catroux"

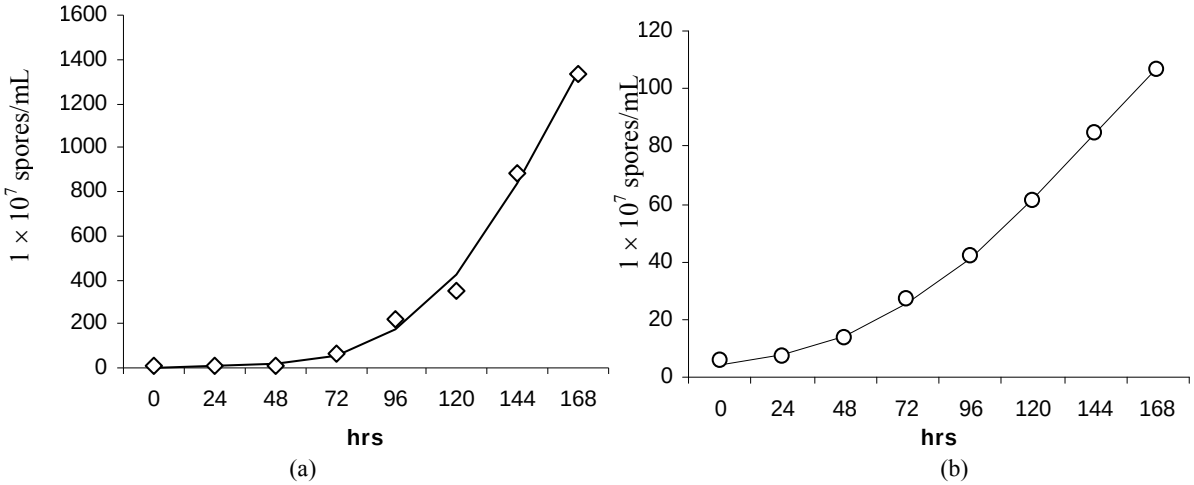

Fig. 3 Adjustement curves of accumulated spore production using the Richards model. Camarón medium, isolate ATCC26854 (a) and Catroux medium, isolate V3 (b).

The points (◊ y ○) correspond to experimental data, and the continuous lines represent estimated data from Richards model.

(B) with V3, which proved to be a typical pattern of sporulation. From 0 h to 36 h ATCC has a lag phase, an exponential phase from 48 h to 72 h, and maximum sporulation is observed (1.38×10^{10} spores/mL) from 96 h to 168 h, while with V3 an accelerated sporulation is observed at 24 h and maximum production of spores occurs at 168 h (near to 1.2×10^8).

3.5 Spore Production

The ANOVA for both isolates, with $\alpha = 0.05$, proves that at least one medium culture produce different results than the rest. The comparison of averages show that for V3 (Table 2), the media Catroux, TKI and Camarón are statistically similar, although in the medium. Camarón maximum production is reached 24 h earlier (120 h) than the other two media (144 h). For the isolate ATCC26854 (Table 3), the culture media which allowed the highest production of spores were Camarón and Minerales, but the Camarón medium reaches maximum production 24 h earlier (144 h) than Minerales. For V3, the maximum production of spores after seven days corresponds to approximately 46 times the concentration of the initial inoculate, whereas in the best medium for the isolate ATCC26854, the maximum production was approximately 1,000 times the concentration of the initial inoculate (Table 3).

The spore production potential differs between both isolates. The Scheffe's test for comparing averages shows that the isolate V3 has a lower production potential compared with the isolate ATCC (Table 4). Isolate V3 seems to differ from the ATCC26854 isolate in its short or absent lag phase and lower production of spores.

3.6 Comparison of Model Parameters

The fit of the spore production curves was higher than 0.99 (r^2) for all the media with both *L. lecanii* isolates. The effect of each medium was compared by the confidence intervals of the different parameters of the model (Table 5). The isolate ATCC differs with respect to maximum sporulation (parameter *a*) and rate of sporulation (parameter *c*), but there are no differences between Paris, Pulgón and Camarón media with respect to the form of the curve (parameter *d*) (Table 6). For V3, the highest spore production was similar ($\alpha = 0.05$) in Camarón, Catroux, and TKI; similarly, the comparison of the means of the confidence intervals of the estimated parameters of the model indicate that sporulation rates and the form of the curves are similar. Nevertheless, the medium TKI differs from Camarón with respect to *a*, *c*

Table 5 Estimated parameters from Richards model and the correlation coefficients of the response variable; spore production of both isolates of *L. lecanii* in different liquid media cultures.

Culture media	ATCC26854					V3				
	r^2	*a*	*b*	*c*	*d*	r^2	*a*	*b*	*c*	*d*
DS	0.9955	4,827.73	1.00	0.01	0.20	0.9995	50.44	3.55	0.03	1.57
Minerales	0.9932	14,023.56	1.65	0.01	0.24	0.9994	106.97	2.01	0.02	0.68
Conidias	0.9958	7,162.04	1.22	0.01	0.22	0.9926	9.14	12.67	0.11	2.28
Pulgón	0.9996	1,335.29	3.22	0.03	0.42	0.9996	122.68	1.94	0.02	0.61
Camarón	0.9974	2,695.22	3.45	0.03	0.45	0.9994	103.00	8.45	0.06	2.54
TKI	0.9917	1,076.97	-0.05	0.01	0.10	0.9996	116.27	4.69	0.03	1.37
Paris	0.9975	1,518.96	3.33	0.03	0.43	0.9996	134.69	2.20	0.02	0.66
Catroux	0.9959	792.09	-1.18	0.01	0.05	0.9997	180.19	1.96	0.02	0.55
Jenkins-Prior	0.9977	2,570.26	0.47	0.01	0.15	0.9997	93.78	2.87	0.02	0.95
Agua	0.9970	3,454.10	1.04	0.01	0.21	0.9993	182.39	-0.09	0.01	0.17
Jackson	0.9943	1,616.09	-0.05	0.01	0.11	0.9993	61.58	1.38	0.02	0.63
Trealosa	0.9994	1,043.75	4.40	0.03	0.62	0.9973	47.91	4.37	0.04	1.83
Adamek	0.9951	7,024.15	1.28	0.01	0.23	0.9996	135.22	2.50	0.02	0.77

r^2 = Correlation coefficient. *a*, *b*, *c* and *d* are the estimated parameters from the Richards model. *a* = curve maximum height, *b* = adjustment constant, *c* = growth rate, and *d* = curve form parameter.

Table 6 Comparison by confidence intervals of the Richards model parameters.

Comparison			Parameter						Conclusion		
			a		*c*		*d*		*a*	*c*	*d*
			-	+	-	+	-	+			
ATCC26854											
DS	vs	Pulgón	1,477.21	5,507.66	-0.02	-0.02	-0.36	-0.08			
DS	vs	Camarón	63.63	4,201.39	-0.02	-0.01	-0.39	-0.12			
Minerales	vs	Pulgón	6,051.38	19,325.16	-0.02	-0.02	-0.30	-0.05			
Minerales	vs	Camarón	4,637.80	18,018.88	-0.02	-0.01	-0.30	-0.09			
Pulgón	vs	Camarón	63.63	4,201.39	0.00	0.01	-0.10	0.03			*
Paris	vs	Minerales	-19,161	-5,848.39	0.02	0.02	0.06	0.32			
Paris	vs	Pulgón	124.41	242.93	0.02	0.00	-0.06	0.09			*
Paris	vs	Camarón	-6,472.53	-1,063.35	0.00	0.00	-0.09	0.05			*
V3											
Camarón	vs	TKI	-11.85	38.41	-0.05	0.00	-2.73	0.40	*	*	*
Camarón	vs	Catroux	33.10	121.30	-0.06	-0.01	-3.27	-0.72			
Camarón	vs	Agua	18.93	139.85	-0.07	-0.02	-3.62	-1.12			
TKI	vs	Catroux	-117.11	-10.74	0.00	0.03	-0.16	1.81			*
TKI	vs	Agua	-135.66	3.43	0.00	0.04	0.24	2.16	*		
Catroux	vs	Agua	-86.32	90.71	0.00	0.02	-0.29	1.04	*		*

a, *b*, *c* and *d* are the Richards model parameters. *a* = maximum height of the curve, b = adjustment constant, *c* = growth rate, and *d* = curve form parameter. The confidence intervals were obtained at 95% confidence; if zero is between the negative and positive, there are no differences (*), otherwise, the conclusion is that there are differences (blank space).

and *d* parameters, and from Catroux with respect to *a* and *c* parameters. The pattern of sporulation of V3 and ATCC26858 does not differ significantly (Table 6).

4. Discussion

The nutrimental conditions of the culture media strongly influence the production of spores of both isolates of *L. lecanii*. The results of our study corroborate similar findings about the effect of nutrimental conditions in the production of spores of *L. lecanii* and other Hyphomycetes fungi [13, 20, 24, 27, 32-34]. The lag phase of ATCC (48 h) was very

long compared with the one observed in V3, which begins the logarithmic phase 24 h after inoculation. In spite of the speed of sporulation of V3, its sporulation potential does not compare with that of ATCC, which 72 h after inoculation reached a spore yield of 1×10^9 spores. This shows that each isolate has different nutritional requirements and that the spore production potential of each of them is also different.

Considering the size and form of the spores, it is possible to state that 100% of the spores produced in the 13 liquid media correspond to conidia and not to blastospores. The average sizes of the conidia agree with what was reported in the studies on solid fermentation [20, 22, 33]; the studies in Ref. [20] and Ref. [33] report a mean size of 5μm with a range of 2.5 μm to 11 μm, whereas the study in Ref. [22] reports spores of between 1.2 μm and 10.8 μm, with a mean size which varies between 3.5 μm and 7.5 μm depending on the isolate. The isolates used in this investigation differ in size; the V3 can be considered to be within the *L. lecanii* group with big spores (5.28-14.29 μm), while the ATCC26854 corresponds to a group with small spores (2.73-6.99 μm).

4.1 Spore Production in Different Media

Conidia were produced in the thirteen media used in this research. The liquid culture with the highest concentration of spores from ATCC26854 was the supplemented medium Camarón with a pH of 6.5 and a spore yield higher than 5×10^9 spores/mL. In the Paris, Jenkins-Prior and Minerales media reported for blastospores production of *B. bassiana*, and conidia of *M. anisopliae* and *P. fumosoroseus*, the isolate ATCC26854 had high concentrations of conidia (3.34×10^9, 3.45×10^9 and 4.69×10^9 conidia/mL respectively). In the rest of the liquid media, the production of spores was within a range of less than 2.7×10^9. In the case of the isolate V3, maximum sporulation occurred at 144-168 h, was higher than 12×10^7 spores/mL, except in the DSB (7.3×10^7 spores/mL), Jackson (7.3×10^7 spores/mL) and Conidia (0.33×10^7 spores/mL) media. The spore yield of this isolate in the best media was higher than 2.2×10^8 spores/mL at 144 h, except in "Camarón", which reached maximum yield 24 h earlier.

4.2 Effect of the Media Components

The medium supplemented with shrimp seems to favor sporulation in both isolates of *L. lecanii*. High levels of amino acids and minerals reported in the shrimp wastes [29, 35, 36] seem to favor the development and sporulation of this fungus. *L. lecanii*, like *B. bassiana*, produces chitinases, which break down chitin and give place to the monomer N-acetyl-glucosamine (GlcNAc) used as nitrogen and carbon source [17]. According to these authors [29, 35, 36], GlcNAc has proven to be better than other media such as yeast-peptone-glucose (YPG), among others, for the production of submerged conidia. These observations can be useful for tailoring biological control agents against specific pests [37].

The liquid media in which a higher concentration of spores was observed included mineral ingredients; these media were: the nutritious broth Minerales ($4.6 \pm 0.26 \times 10^9$ spores/mL) and Paris ($3.3 \pm 3.83 \times 10^9$ spores/mL) for ATCC, and Catroux ($2.3 \pm 1.72 \times 10^8$ spores/mL) and TKI ($2.2 \pm 0.26 \times 10^8$ spores/mL) for V3. Nevertheless, ATCC had a high spore yield in the Jenkins-Prior medium, which only contains saccharose (30 g) and yeast extract (20 g); in similar way, the isolate V3 produced $1.8 \pm 3.83 \times 10^8$ conidia/mL in the medium "Agua" (30 g of dextrose and 30 g of yeast extract), with no significant differences compared to mineralized media. The Jackson medium, in spite of having a high mineral content, did not favor a significant spore production in any of the isolates.

Nitrogen sources [38] have an important effect on the growth and sporulation process. According to these authors [38], maximum sporulation is obtained in media with KNO_3, NH_4SO_4, NH_4NO_3, γ aminobutiric acid, aspartic acid, lisine and treonine. In

our study, these components were in the media in which maximum spore production was observed (Catroux, TKI, Paris, Minerales), or were added as complements in the yeast extract or peptones. Similar results are reported by Ref. [9] and Ref. [39], who mentioned that the sporulation of *B. bassiana* was favored by the presence of nitrate. In the present study, the presence of phosphate (KH_2PO_4) did not seem to have a negative effect on conidia development, as was mentioned in Ref. [17]. The media Catroux, TKI, Paris and Minerales, which were considered as favorable for the sporulation of ATCC and V3 *L. lecanii* isolates, have a high concentration of potassium phosphate.

The Scheffe test demonstrated that, for V3, the media with yeast extract produced more conidia (Catroux, Adamek, Jenkins-Prior and Agua) than the media with beef peptone and hydrolyzed casein; unlike what happens with the isolate ATCC26854, which presented higher levels of conidia in media with beef peptone and hydrolyzed casein.

4.3 Sporulation Model

Although the literature recognizes that fungus growth in media with disaccharides or oligosaccharides requires digestive enzyme synthesis, and, therefore, a period of induction [40], the media did not show differences (in Scheffe tests) regarding the production of spores as far as glucose or saccharose were used as C source for both isolates. Nevertheless, it is assumed that the time the fungus takes to feed is prolonged, as reflected by the parameter "*c*" (rate of growth) of the Richards model which shows that the Jenkins-Prior and Agua (with saccharose) media have low sporulation rates with both isolates.

The three sigmoid growth models tested describe the sporulation dynamics, fitting the data very well; determination coefficients were very similar and the residues showed a typically erratic pattern, which demonstrates the suitable parameterization of the models [21]. The Richards model was selected, since its fourth parameter suitably describes the form of the curve. This model explains the delayed lag phase (48-36 h) better than the Logistic (sigmoid symmetrical) model, or the Gompertz model (absence of lag phase). Estimates of parameter *d* show certain similarities, mainly with isolate ATCC (Table 4), which fortifies the use of this model with a fourth parameter of shape, that represent and fit the sporulation phenomenon.

5. Conclusions

We can produce conidia and not blastospores with these isolations and culture mediums. The liquid culture with the highest concentration of spores from ATCC26854 was the supplemented medium Camarón with a spore yield higher than 5×10^9 spores/mL. The composition of the Camarón and Jenkins-Prior media is simple and inexpensive. Thanks to the modeled process of spore production in liquid fermentation, it is possible to select the medium Camarón which presents higher spore production with both isolates, allowing a high rate of sporulation (parameter "*c*", of more than 0.06×10^7 spores/mL·h). For this reason, the Camarón medium can be recommended as the most suitable medium for the commercial production of conidia of *L. lecanii* in liquid fermentation.

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