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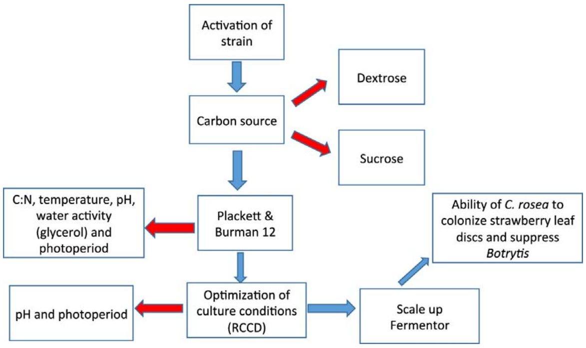
Optimizing the mass production of *Clonostachys rosea* by liquid-state fermentation

André Luiz de Andrade Carvalho[a], Larissa Castro de Rezende[a], Lúcio Bertoldo Costa[b], Bernardo de Almeida Halfeld-Vieira[c], Zayame Vegette Pinto[b], Marcelo Augusto Boechat Morandi[c], Flávio Henrique Vasconcelos de Medeiros[a], Wagner Bettiol[c,*]

[a] *Department of Plant Pathology, Universidade Federal de Lavras, CP 3027, Lavras, MG 37200-000, Brazil*
[b] *Faculdade de Ciências Agronômicas, Universidade Estadual Paulista “Júlio de Mesquita Filho”, 18.610-307 Botucatu, SP, Brazil*
[c] *Embrapa Meio Ambiente, CP 69, 13820-000 Jaguariúna, SP, Brazil*

GRAPHICAL ABSTRACT

Flowchart of the studies for optimization and mass production of *Clonostachys rosea* in liquid medium.



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ABSTRACT

Optimization of the culture medium and the entire mass production process for various fungi in liquid medium has been studied. However, the technology is not well developed for *Clonostachys rosea*, a biocontrol agent against various plant pathogens due to its antagonistic capacity to act as a hyperparasite, compete for nutrients and space, and induce plant resistance to pathogens. In this study, we aimed to optimize the culture medium and to standardize parameters that may interfere with the production of *C. rosea* conidia in a liquid-state fermentation system. Culturing was performed in 250-mL Erlenmeyer flasks shaken for 7 days, followed by planned experimental methodology to reduce the number of analyses and consumable costs. Benchtop bioreactor tests with the optimized medium were performed. Glucose and sucrose were evaluated as carbon sources. Initially, the effects of temperature, pH, photoperiod, carbon:nitrogen ratio and water activity on inoculum production were evaluated, with the pH and photoperiod being factors that contributed to conidial production. Optimization of the fermentation conditions was performed using a central composite rotational design (CCD) with a wider range of pH values and photoperiods. The remaining variables were fixed according to the previous assay. Colony-forming unit (CFUs), biomass production and conidial viability were evaluated, and glucose was used as a carbon source to enhance conidial production. The optimized conditions that resulted in a maximum

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[*] Corresponding author.
E-mail address: wagner.bettiol@embrapa.br (W. Bettiol).

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yield of conidia (1.78×10^7 conidia mL^{-1}), dried biomass (0.558 g) and CFUs (5.15×10^6 CFUs mL^{-1}) were a pH value of 4 and a photoperiod of 12 h.

1. Introduction

Clonostachys rosea (sin. *Gliocladium roseum*: teleomorph *Bionectria ochroleuca*; Schroers et al., 1999) can suppress the sporulation of several plant pathogens by competing for saprophytic growth, which limits the colonization of senescent tissues by the pathogen. Specifically, this bioagent suppresses *Botrytis cinerea* sporulation and infection by competing for nutrients and colonizing wounds (Peng and Sutton, 1991; Sutton and Peng, 1993; Sutton et al., 1997). For example, *C. rosea* was found to be more efficient than fungicides in controlling Botrytis blight in strawberries in field trials (Cota et al., 2008a,b), and due to its effectiveness, reliability and security, growers worldwide have adopted this biocontrol agent. *C. rosea* also has other advantages, such as easy inoculum production in grains, low allergy risk in workers exposed to the product and low ecological risk due to its ubiquitous distribution in plants and soil (Schroers, 2001; Sutton and Peng, 1993; Toledo et al., 2006).

Although it is well documented that *C. rosea* can serve as a biological control agent against plant pathogens, mass production of inoculum relies on solid-state fermentation (Zhang et al., 2013; Zhang et al., 2015). The mass production of *C. rosea* in solid-state fermentation (SSF) of cereal grains is a widely used and well-known process. However, SSF is time- and space-consuming procedure, which makes it expensive. A promising alternative to overcome the limitations of SSF is liquid-state fermentation (LSF), with low-cost, large-scale production that is necessary for agricultural use. Multiplication of conidia in liquid media represents the most efficient and economic method for the mass production of bioagents (Jakubíková et al., 2006; Watanabe et al., 2006). A close relationship exists between the inoculum produced and the composition of the culture medium, which must include carbon and nitrogen sources, mineral salts and growth factors (Soper and Ward, 1981). Indeed, the medium influences the type, format and quantity of propagules produced.

Production in submerged culture has many advantages compared to solid substrates: it is easier to control fermentation parameters, such as pH and temperature, allows for better control of contamination and demands less space and labor (Mascarin et al., 2015).

As it enables producers to conduct experiments with lower material costs, culturing using liquid medium in Erlenmeyer flasks is widely used in the optimization of biotechnological processes before the implementation of bioreactors (Buchs, 2001). The Plackett-Burman design (PBD) and response surface methodology (RSM) have been employed for the optimization of such bioprocesses (Montgomey, 2005). These experimental designs allow for the evaluation of several influencing factors as well as interactions between them, which enables the selection of the best conditions to optimize the culture medium to provide maximum conidial production, with reductions in labor, time and costs (Montgomey, 2005).

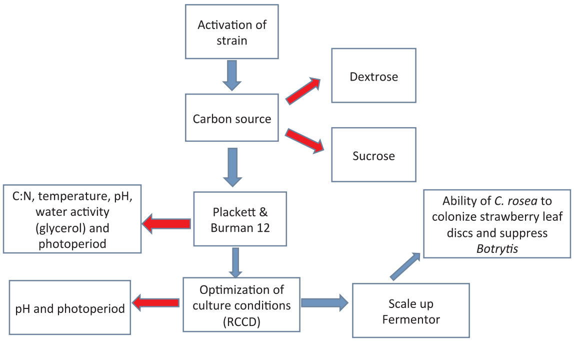

Fig. 1. Flowchart of the studies for optimization and mass production of *Clonostachys rosea* in liquid medium.

Considering the growing market for this biocontrol agent in Brazil and other countries, the objective of this work was to optimize the culture medium [carbon:nitrogen (C:N) ratio, pH and water activity] and growth conditions (temperature and photoperiod) for maximum *C. rosea* conidial production. The efficacy of the growth medium and the optimized culture conditions were tested in a benchtop bioreactor for *C. rosea* conidial production; the obtained *C. rosea* inoculum was also assessed with regard to its potential for gray mold (*B. cinerea*) biocontrol using strawberry leaf discs.

2. Materials and methods

The *C. rosea* strain LQC 62 (sequence is deposited in the NCBI Sequence Database under accession number MG489966) used in these studies was isolated from rose crops at Viçosa, Minas Gerais State, Brazil. This strain was previously selected as tolerant to UV-B radiation when compared to other strains (Costa et al., 2012). The isolate was deposited at the Embrapa Environment's collection of microorganisms. The fungus was grown in Potato-Dextrose-Agar (PDA) (Acumedia Manufacturers, Michigan, USA) in plates (polystyrene, 90 × 10 mm, Pleion) at 25 ± 2 °C and 12 h light/12 h dark for 21 days. The conidia were suspended in Tween 80 solution with distilled water (0.01% v/v), and the concentration was adjusted to 3.0×10^6 conidia mL^{-1} in a Neubauer chamber. Fig. 1 shows the flowchart of the studies for optimization of the mass production of *C. rosea* in liquid medium.

2.1. Carbon sources for *C. rosea* conidial production

The basic culture broth used was a modified semi-synthetic Czapek-Dox medium, with peptone from casein as a nitrogen source (12% N) and 10 g L^{-1} sucrose (42.1% C) or dextrose (40% C) as carbon sources. The basal medium was composed of 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$, and 0.02 g $FeSO_4$. The C:N ratio (20:1, 110:1 and 200:1), glycerol (water-related activity) and pH were in accordance with each treatment (Matrix PBD 12). The initial pH was adjusted before sterilization with 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} H_3PO_4.

Optimization of the culture conditions was performed using a Box-Behnken central composite design (CCD). Statistical designs were applied to investigate the effects of temperature, pH, photoperiod, the C:N ratio and water activity (glycerol) (Tables 1 and 2), on the production of conidia, CFUs and dry fungal biomass. PBD 12, which is a design that provides information on the importance of the effect on the results and if the chosen track is the most appropriate one and indicates which

Table 1
Values of factors and levels (low, central and high) of the experimental planning Plackett-Burman Design 12 (PBD 12).

Factors	Code	Level		
		−1 (low)	0 (central)	1 (high)
pH	X_1	3.5	6	8.5
Temperature (°C)	X_2	20	24	28
Photoperiod (h)	X_3	0	12	24
C:N ratio	X_4	20:1	110:1	200:1
Glycerol (v/v)	X_5	0	1	2

Table 2
Effect of pH, temperature, photoperiod, C:N ratio and glycerol on concentration of conidia mL^{-1}, number of CFUs mL^{-1} and dry biomass (g) of *Clonostachys rosea* grown in liquid medium with the addition dextrose or sucrose as a carbon source.

Trial	Factors					Conidia mL^{-1}		CFUs mL^{-1}		Dry biomass	
	X1	X2	X3	X4	X5	D	S	D	S	D	S
1	1	−1	1	−1	−1	1.01×10^{7}	2.05×10^{6}	7.91×10^{6}	3.24×10^{6}	0.327	0.412
2	1	1	−1	1	−1	2.50×10^{5}	3.14×10^{6}	1.19×10^{6}	3.58×10^{6}	0.339	0.412
3	−1	1	1	−1	1	1.00×10^{7}	1.17×10^{6}	9.07×10^{6}	7.90×10^{6}	0.608	0.659
4	1	−1	1	1	−1	1.35×10^{6}	8.11×10^{5}	2.05×10^{6}	1.11×10^{5}	0.319	0.340
5	1	1	−1	1	1	6.25×10^{5}	5.42×10^{5}	1.78×10^{6}	3.47×10^{5}	0.429	0.478
6	1	1	1	−1	1	5.19×10^{6}	2.98×10^{6}	4.42×10^{6}	4.95×10^{6}	0.657	0.520
7	−1	1	1	1	−1	1.52×10^{7}	7.30×10^{6}	1.30×10^{7}	9.97×10^{6}	0.478	0.366
8	−1	−1	1	1	1	9.08×10^{6}	5.98×10^{6}	1.27×10^{7}	1.14×10^{6}	0.520	0.401
9	−1	−1	−1	1	1	1.14×10^{7}	4.51×10^{6}	8.14×10^{6}	6.30×10^{6}	0.385	0.499
10	1	−1	−1	−1	1	8.75×10^{5}	2.81×10^{6}	8.64×10^{5}	5.78×10^{5}	0.410	0.509
11	−1	1	−1	−1	−1	5.90×10^{6}	5.99×10^{6}	2.42×10^{6}	9.19×10^{6}	0.399	0.452
12	−1	−1	−1	−1	−1	1.18×10^{7}	3.58×10^{5}	7.18×10^{6}	4.01×10^{5}	0.391	0.381
13	0	0	0	0	0	1.02×10^{7}	4.12×10^{6}	1.03×10^{7}	4.92×10^{6}	0.500	0.438
14	0	0	0	0	0	3.61×10^{6}	3.03×10^{6}	5.8×10^{6}	4.57×10^{6}	0.397	0.507
15	0	0	0	0	0	1.16×10^{7}	3.27×10^{6}	5.86×10^{6}	2.32×10^{6}	0.426	0.475

X_1 = pH; X_2 = Temperature; X_3 = photoperiod; X_4 = Carbon:Nitrogen ratio; X_5 = Glycerol (%). D = dextrose and S = sucrose. The values of these parameters were presented in Table 1.

direction to go in the next planning step, was used. A CCD was performed with a 2^2 factorial for finding the optimal region, enabling optimization of the culture conditions (Rodrigues and Iemma, 2009).

A PBD was also used for choosing the best carbon source. Assays were assembled using dextrose ($C_6H_{12}O_6$) or sucrose ($C_{12}H_{22}O_{11}$). This study was performed in 250-mL Erlenmeyer flask containing 90 mL of culture medium plus 10 mL of *C. rosea* suspension (3×10^6 conidia mL^{-1}). After seven days of incubation in a shaker at 160 rpm, the conidial concentration, CFUs and fungal biomass were determined. The flasks were placed in an ultrasonic bath for 5 min before collection of a 1-mL aliquot of culture. The aliquot was transferred to a tube containing 9 mL of saline solution with Tween 80 (PA NaCl: 9 g; distilled water: 1000 mL and Tween 80: 1 mL), and serial dilutions up to 10^{-4} were performed. Conidial quantification was performed in a Neubauer chamber. For CFU determination, 100 µL of the 10^{-4} dilution was transferred to a Petri dish containing PDA + Triton (1 mL/1000 mL) and spread with a Drigalski spatula over the plate surface in 5 replicates for each treatment. The colony counts were performed after 72 h of incubation. For conidial germination evaluation, 5 aliquots of 15 µL of the 10^{-4} dilution were transferred to Petri dishes with PDA medium. The plates were incubated at 25 °C for 24 h, and germination was interrupted with 0.05% trypan blue in lactophenol. Conidia were observed at 400× magnification. Conidia with a germ tube longer than the major diameter of the conidia were considered germinated. A total of 100 conidia per treatment were evaluated. To evaluate biomass production, the culture medium was filtered through filter paper, and the obtained mycelia were dried in an oven at 55 °C until a constant weight was attained. The difference between the weight of each sample and the filter paper without the mycelia was recorded as the result.

2.2. Assays for optimization of the culture medium and conditions with dextrose as the carbon source

Five factors, the C:N ratio, pH, water activity, temperature and photoperiod (Table 1), were investigated in tests for optimization of the culture medium. This followed PBD 12, which was composed of 12 tests + 3 central points and included 15 experiments (Table 2). These tests were based on RSM (Rodrigues and Iemma, 2009) using dextrose as the carbon source, which was selected previously. This is a plan that provides information on the importance of the effects on the results and if the selected track is the most appropriate, indicating the direction to follow and a CCD with 2^2.

Assays to evaluate the optimal conditions for the highest production of conidia using a previously selected C source were performed in 250-mL Erlenmeyer flasks, as described above. Light was provided by 36 W daylight lamps. To simulate a continuous-dark treatment, the flasks were thoroughly wrapped with aluminum foil.

After culture medium optimization, the temperature, C:N ratio and glycerol concentration (Table 2) were fixed to maximize conidial production. Optimization of the culture conditions selected in PBD 12 was determined with RCCD and a composite of 11 trials for 2 variables (pH and photoperiod) at each of 5 levels (Table 2). The assays were performed and evaluated using the same methodology as described above.

2.3. Scale-up: laboratory benchtop bioreactors

The optimized culture medium and fermentation conditions selected using Erlenmeyer flasks were evaluated in a laboratory scale-up assay using a bioreactor (Microferm fermenter New Brunswich Scientific MF-105, Edison, NJ) of 10 L, with temperature and aeration control. Czapek-Dox modified medium was used with the best variable values according to the results obtained in the CCD, with a C:N ratio of 200:1, 1.12 g of casein peptone, and an initial pH of 4. For each batch, 6.3 L of medium was prepared and inoculated with 700 mL of conidial suspension at 3×10^6 conidia mL^{-1}. The temperature was set to 25 ± 1 °C. The stir speed was set to 160 rpm with 1 vvm aeration (volume of air per minute per volume of culture medium) and a photoperiod of 12 h. From the beginning of the experiment until the 7th day, a 10-mL aliquot was taken from the culture every day, and parameters (pH, conidial concentration, CFUs, conidia germination and dry weight) were evaluated. At the end of the trial, the produced inoculum was evaluated for its ability to colonize strawberry leaf discs, control gray mold and suppress *B. cinerea* sporulation.

2.4. Reduction in B. cinerea sporulation on strawberry leaf discs treated with C. rosea conidia produced in a benchtop bioreactor

The ability of the *C. rosea* inoculum produced in the benchtop bioreactor to colonize strawberry leaf discs and suppress the sporulation of *B. cinerea* strain LQC 150 was evaluated following the methodology proposed by Morandi et al. (2000). Leaf discs (1 cm in diameter) of 60-day-old strawberry plants (cv. Camarosa) were surface sterilized in 70% ethanol (1 min) followed by 2% sodium hypochlorite (1 min) and rinsed three times in sterile distilled water. Ten discs per plate were transferred to paraquat chloramphenicol agar medium (PCA) in Petri dishes (Peng and Sutton, 1991), and 20 µL of *C. rosea* conidial suspension at 10^5 or 10^6 conidia mL^{-1} was added to each disc. Half of the

Table 3
Estimated values of the effects and *p* for pH, temperature, photoperiod, C:N ratio and glycerol in the production of *Clonostachys rosea* conidia.

Variable	Effect value	*p*-value
pH	−1.77847	.002065
Temperature	−0.60032	.182906
Photoperiod	1.11444	.025257
C:N	−0.59486	.186536
Glycerol	−0.01124	.979039

discs treated with *C. rosea* were then treated with an aliquot of *B. cinerea* (10 μL at 10^5 conidia mL^{-1}). The discs were incubated at 25 ± 2 °C (12 h light/12 h dark), and the growth and sporulation rate of *C. rosea* and the pathogen were estimated on the 7th and 10th days under a stereomicroscope (10× magnification) following the scale represented by the disc area covered by *C. rosea* conidiophores. The evaluation was as follows: 0 = 0% (0%); 1 = 2% (1–3%); 2 = 5% (4–6%); 3 = 10% (7–13%); 4 = 20% (14–27%); 5 = 40% (28–52%); 6 = 70% (53–87%); and 7 = 94% (88–100%) (Morandi et al., 2000). An assessment of reductions in *B. cinerea* sporulation was performed, and measurements were taken following a scale of notes for the area of the discs covered with *B. cinerea* conidiophores, as follows: 0 = 0% (0%), 1 = 2% (1–3%), 2 = 5% (4–6%), 3 = 10% (7–12%), 4 = 20% (13–26%), 5 = 40% (27–53%), 6 = 65% (54–76%), and 7 = 90% (77–100%) (Peng and Sutton, 1991). The area under the colonization progress curve (AUCPC) and the area under the sporulation progress curve (AUSPC) were calculated for both fungi (Shaner and Finney, 1977).

2.5. Experimental design and data analysis

All assays were performed in triplicate and repeated 3 times. Each experiment was conducted in a completely randomized design, with 3 replicates. For evaluations, 3 replicates were performed on 1 plate, and each plate contained 10 leaf discs. The data from the 3 experimental repetitions invariably resulted in data that had significance in the same range; therefore, the data were grouped for analyses. Statistical computations were performed using Statistica 10.0. For AUIPC and AUSPC, Statistical Analysis Systems (SAS Institute Inc., Cary, NC) was used. Data for fungal germination and sporulation were examined using analysis of variance (ANOVA). AUIPC and AUSPC were compared using Tukey's test ($p \leq 0.05$).

The effects of the culture medium and conditions on conidial production were analyzed using CCD, and ANOVA was performed with STATISTICA 10.0. For estimation of the pure error, central points (0) were used. The polynomial model including linear and quadratic coefficients was used to calculate the predicted response. The significance of the regression model parameterized in CCD was determined using Student's *t* test at a 0.05 probability level.

3. Results

3.1. Carbon sources for C. rosea conidial production

The addition of dextrose as a carbon source promoted the best conidial production in trials 1, 3, 4, 6, 7, 8, 9, 12, 13, 14 and 15 (Table 2). Superior production of conidia was only observed in trials 2, 10 and 11, with sucrose addition. The experimental condition that promoted the best conidial production (1.52×10^7 conidia mL^{-1}) was trial 7 (pH = 3.5, temperature 28 °C, photoperiod 24 h, C:N 200, without glycerol and with dextrose as a C source). Using sucrose as a carbon source and fixing all the other variables, the highest conidial concentration was obtained for trial 7 had (7.3×10^6 conidia mL^{-1}), though the result was two times less than that using dextrose (Table 2).

Dextrose resulted in the highest number of CFUs for trials 1, 3, 4, 7, 8, 9, 10, 12, 14 and 15 (Table 2), with the highest production observed in trial 7 (1.3×10^7 CFU mL^{-1}). Sucrose addition in trial 7 resulted in the highest CFUs for this C source (9.97×10^6 CFU mL^{-1}) (Table 2). Dry biomass (Table 2) was similar regardless of whether dextrose or sucrose was used as the carbon source. The maximum fungal biomass produced was 0.657 g with dextrose (trial 6) and 0.659 g with sucrose (trial 3).

3.2. Optimization of the culture medium and fermentation conditions (C:N ratio, pH, water activity, temperature and photoperiod) with dextrose as the C source in liquid medium using the Plackett-Burman 12 design

The highest conidial concentration was observed in trial 7 (pH = 3.5, temperature = 28 °C, constant light, C:N = 200, without glycerol). Under these conditions, *C. rosea* produced 1.52×10^7 conidia mL^{-1} (Table 2). Trials 9 and 12, with parameters similar to the 7th and 9th trials (pH = 3.5, C:N = 200 and without glycerol addiction), showed the second highest conidial production (1.14×10^7 mL^{-1} and 1.18×10^7 mL^{-1}). The lowest conidial productions was observed in trials 2 and 5 had (2.5×10^5 mL^{-1} and 6.25×10^5 mL^{-1}, respectively) (Table 2).

The pH and photoperiod parameters were statistically significant, with *p* values < .002 and < .025, respectively (Table 3). These results are also illustrated in the Pareto diagram (Fig. 2A), where the bars that extend beyond the reference line are considered significant, indicating that the next CCD is 2^2. Estimates of the effect of pH, temperature, the C:N ratio and glycerol had negative values, allowing the use of lower values for each variable in the CCD (Table 3 and Fig. 2A).

The pH, photoperiod and temperature variables were significant ($p \leq .001$) with regard to CFUs, as shown in the Pareto diagram (Fig. 2B). The highest CFU values were observed for trials 7 and 8 and the lowest for trials 2, 5 and 10 (Table 2). An increase in temperature to 28 °C and in pH to 8.5 without a decrease in light resulted in 98.4% *C. rosea* CFUs mL^{-1} for trials 2 and 5.

The highest degree of dry biomass production was achieved under the trial 6 conditions (0.657 g) and the lowest biomass in trial 4 (0.319 g) (Table 2). Temperature, photoperiod and glycerol were significant ($p < .001$) for dry biomass production, as shown in the Pareto diagram (Fig. 2C). Dry biomass increased with temperature in the presence of glycerol.

Once we had determined the temperature (25 °C) and the C:N ratio (200:1) without the addition of glycerol for maximum conidial production, those variables were fixed for the following CCD.

Using the test proposed by the CCD, the inoculum production for the culture conditions selected in the PBD 12 increased from 1.05×10^5 to 1.78×10^7 conidia mL^{-1}. Trials 6 and 2, with an initial pH of 4 and 3.71, respectively, produced the highest amount of conidia (1.78×10^7 and 1.22×10^7 conidia mL^{-1}) and the highest CFUs mL^{-1} (Table 4). In contrast, trials 1 and 5, with an initial pH of 2.29 and 2.0, respectively, produced the smallest amounts of conidia. The pH of the linear term, given by the 95% significance model, was statistically significant for the spore concentration, generating a linear model (Table 5) with $R^2 = 0.7967$. The reparametrized equation $Y_1 = 14.728 + 1.569x_1$ represents the linear model for conidial production as a function of pH, where Y_1 is the predicted response of conidial production and x_1 is the encoded pH value. The linear and quadratic terms for pH were significant (95% significance) for the CFU value. For dry biomass, only the linear term for pH was significant (Table 5). The coefficients of determination of the reparametrized models for CFUs and biomass were $R^2 = 0.90$ and $R^2 = 0.72$, respectively. The equations $Y_2 = 14.876 + 4.054x_1 - 3.173x_2$ and $Y_3 = 0.3601 + 0.1356x_1$ are representative of the reparametrized models for CFUs and biomass as a function of pH, where Y_2 and Y_3 are the predicted response of CFUs and fungal biomass, respectively, and x_1 is the encoded pH value (Table 5).

The settings for spore concentration, CFUs and dry biomass of the predicted models were considered adequate. The boundary surfaces,

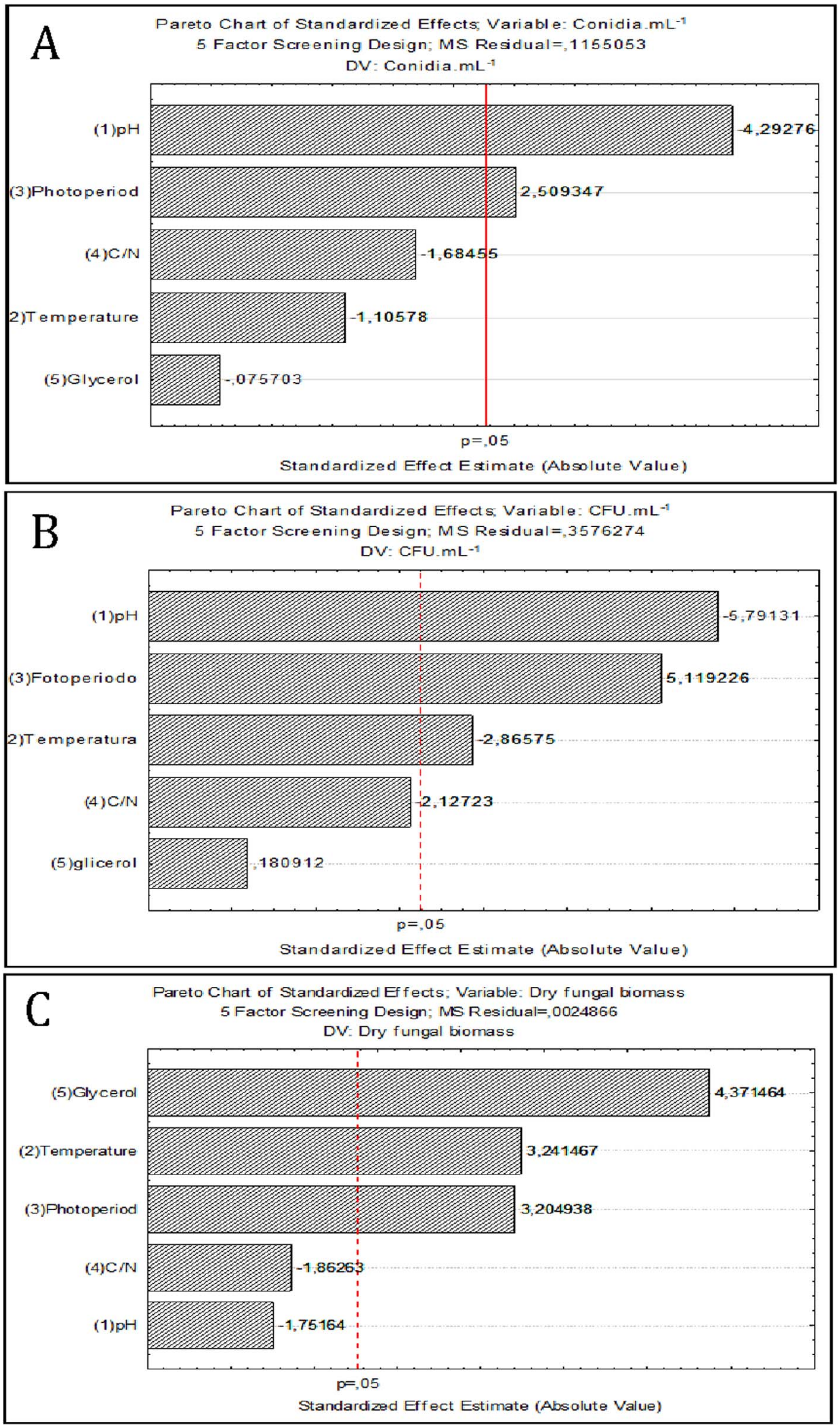

Fig. 2. Pareto diagram demonstrating the significance of the pH, photoperiod, temperature, C:N ratio and glycerol variables in production of conidia (A), CFUs (B) and dry biomass (C) of *Clonostachys rosea* in optimized culture medium.

generated from the models generated from the equations, present the maximal value for the spore concentration outside the studied range (Fig. 3A). The range of values observed in trials with CCD tended to be more to the right, straddling higher pH values. Observing the boundary surface for CFUs (Fig. 3B), the optimal pH and photoperiod ranges were from 3 to 4 and 12 h to 24 h, respectively. Fungal biomass increased with an increase in the pH of the medium (Fig. 3C).

3.3. Scale-up: laboratory benchtop bioreactors

The conidial concentration increased in the first 2 days of fermentation but remained stable until the 6th day. The conidial concentration reached its maximum on the 7th day, with a final concentration of 1.01×10^8 conidia mL^{-1} (Fig. 4A). A similar pattern of maximal CFUs was observed, though with a lower concentration (4.5×10^7 CFUs mL^{-1})

Table 4
Effects of pH and photoperiod on the conidial concentration, colony-forming-units (CFUs) and dry biomass of *Clonostachys rosea* for optimization of culture conditions selected in the PBD 12 with a central composite design (CCD). A composite of 11 trials.

Trial	pH (X1)	Photoperiod (h) (X2)	Conidia mL^{-1}	CFUs mL^{-1}	Dry biomass (g)
1	−1 (2.29)	−1 (4)	1.05×10^5	2.0×10^4	0.194
2	1 (3.71)	−1 (4)	1.22×10^7	3.9×10^6	0.557
3	−1 (2.29)	1 (20)	3.75×10^5	2.0×10^5	0.129
4	1(3.71)	1 (20)	5.88×10^6	3.91×10^6	0.52
5	−1.41(2.0)	0 (12)	1.14×10^5	0	0.153
6	1.41 (4.0)	0 (12)	1.78×10^7	5.15×10^6	0.385
7	0 (3.0)	−1.41 (0)	3.0×10^6	1.97×10^6	0.411
8	0 (3.0)	1.41 (24)	8.88×10^6	2.52×10^6	0.465
9	0 (3.0)	0 (12)	3.0×10^6	1.44×10^6	0.357
10	0 (3.0)	0 (12)	1.05×10^7	3.47×10^6	0.397
11	0 (3.0)	0 (12)	1.20×10^7	1.53×10^6	0.391

Table 5
Coefficient of variation, error, t and *p* values estimated by the regression model parameterized in a central composite design (CCD) for conidial concentration, CFUs and dry biomass.

Factor	Coefficient of variation	Error	t (5)	*p*-value
Conidia concentration				
Average	14.72806	0.224953	65.47178	0.0000
pH (Linear)	1.56901	0.264174	5.93932	0.000218
CFU				
Average	14.87636	0.687990	21.62292	0.0000
pH (Linear)	4.05443	0.579352	6.99821	0.000113
pH (Quadratic)	−3.17260	0.661321	−4.79736	0.001360
Dry biomass				
Average	0.360094	0.024258	14.84406	0.0000
pH (Linear)	0.135616	0.028488	4.76046	0.001029

(Fig. 4B). Germination of the conidia produced in the bioreactor was between 94% and 96%, regardless of the assessment day. Biomass production was 0.63 g 100 mL^{-1} of culture medium. The pH of the culture medium increased during the fermentation period. The initial pH increased gradually, reaching a plateau at 8.27 on the 6th day (Fig. 4C).

3.4. Reduction in B. cinerea sporulation on strawberry leaf discs treated with conidia of C. rosea produced in benchtop bioreactors

C. rosea conidia produced in the optimized medium in benchtop bioreactors were able to effectively colonize strawberry leaf discs. The AUCPC was higher when the conidial suspension was applied at 10^6 conidia mL^{-1} (Fig. 5A). However, sporulation of *C. rosea* on the leaf discs was similar for the 2 concentrations used when analyzing the AUSPC (Fig. 5B). *B. cinerea* colonization and sporulation on the leaf discs were significantly reduced in the presence of *C. rosea* (Fig. 5CD), and 10^5 and 10^6 conidia mL^{-1} of *C. rosea* reduced the incidence of the pathogen on the leaf discs by 32 and 48%, respectively. In addition, pathogen sporulation was reduced by 47% and 58%, respectively, by *C. rosea* at the same concentrations (Fig. 5CD).

4. Discussion

C. rosea exhibits enormous potential as a biofungicide for biological control of plant diseases. However, there are few commercial products based on *C. rosea* (Bettiol et al., 2012). For agricultural use, low-cost, large-scale production is necessary, but mass production of *C. rosea* is currently achieved via solid-state fermentation (SSF). Liquid-state fermentation (LSF) is a promising alternative for overcoming the limitations of SSF. Although optimization of the culture medium and the entire mass production process for various conidial fungi in liquid medium have been studied, this is not a well-developed technology for *C. rosea*.

Recent publications (Sun et al., 2013; Zhang et al., 2013, 2015) have addressed *C. rosea* production in LSF and in two-phase solid fermentation. However, information on the mass production of conidia in submerged fermentation remains scarce. Our studies showed for the first time the feasibility of *C. rosea* conidial production in submerged fermentation. Our results on the optimization of liquid culture medium and the incubation conditions (Figs. 2–4, Tables 1–5) that increase the production of *C. rosea* conidia can contribute to the development of new commercial products to provide not only conidia but also supernatant for improving disease control (Rodriguez et al., 2011; Zhai et al., 2016).

Many reports demonstrate that the carbon source and the C:N ratio of the culture medium greatly affect the growth and sporulation of various fungi (Thomas et al., 1987; Engelkes et al., 1997; Elson et al., 1998; Gao, 2011; Kobori et al., 2015). For *Beauveria bassiana*, Thomas et al. (1987) found that dextrose was more effective as a source of C for spore production in liquid medium, and we found that semi-synthetic Czapek-Dox liquid basic culture broth with dextrose as a carbon source increased conidial production of *C. rosea* by almost 50% compared to sucrose (Table 2). The results indicate that for *C. rosea*, monosaccharides are more efficient at triggering conidiogenesis than disaccharides, likely because they are more easily metabolized. In our study, the Pareto diagram (Fig. 2) indicated negative values for the C:N ratio.

Engelkes et al. (1997) reported that a higher C:N ratio increased sporulation of *Talaromyces flavus*, whereas reduced conidiation of the plant-pathogenic fungus *Helminthosporium solani* was observed (Elson et al., 1998). In addition, Gao (2011) showed that different combinations of carbon and nitrogen sources can lead to different spore yields of *B. bassiana* in liquid fermentation. In the present study, the amounts of carbon and nitrogen were not considered significant during PBD 12, but it was observed that the highest *C. rosea* conidial production occurred at the 200:1 ratio. Overall, carbon and nitrogen sources, as well as the C:N ratio, have important implications for the cost of mass production of microorganisms. Therefore, understanding the nutritional requirements of the microorganism and optimization of the fermentation medium is essential for the viability of a commercial biocontrol product (Gao, 2011; Peng et al., 2014).

Although there are advantages of LSF, an important concern about conidial production in submerged fermentation is abiotic stress tolerance and maintenance of effective antagonistic ability (Jaronski, 2014). Watanabe et al. (2006) observed that the *Trichoderma asperellum* structures produced in solid fermentation had a greater tolerance to desiccation than those produced in liquid medium. Sriram et al. (2011) reported that despite decreasing the water activity of the medium, glycerol in the culture medium increased the shelf life of formulations based on *Trichoderma harzianum*. For *C. rosea* LQC 62 strain, the addition of glycerol to the culture medium was considered non-significant and had a negative effect on conidial production, increasing dry biomass rather than conidia (Table 2). In addition to the role of glycerol in regulating water activity, adding it to the culture medium can also alter the C:N ratio when it is used as a carbon source for fungal growth in submerged culture (Meinicke et al., 2012). The negative effect of glycerol on conidial production led us to infer that *C. rosea* utilized the amended glycerol as a supplementary carbon source, with changes to the suitable C:N ratio and a reduction in conidial production, which benefits mycelial biomass. These studies may be performed or adapted in the future, indicating their usefulness for lowering concentrations or adding later fermentation stages to prime conidia for drought tolerance, even in formulation stages.

In addition to reducing the number and tests, by using the CCD design, which allows for evaluation of the interference of variables in the entire process and interactions among them, it was observed that pH was the variable that had the greatest influence on the entire process (Tables 2 and 4). The initial pH values of 3.71 to 4.0 resulted in the best

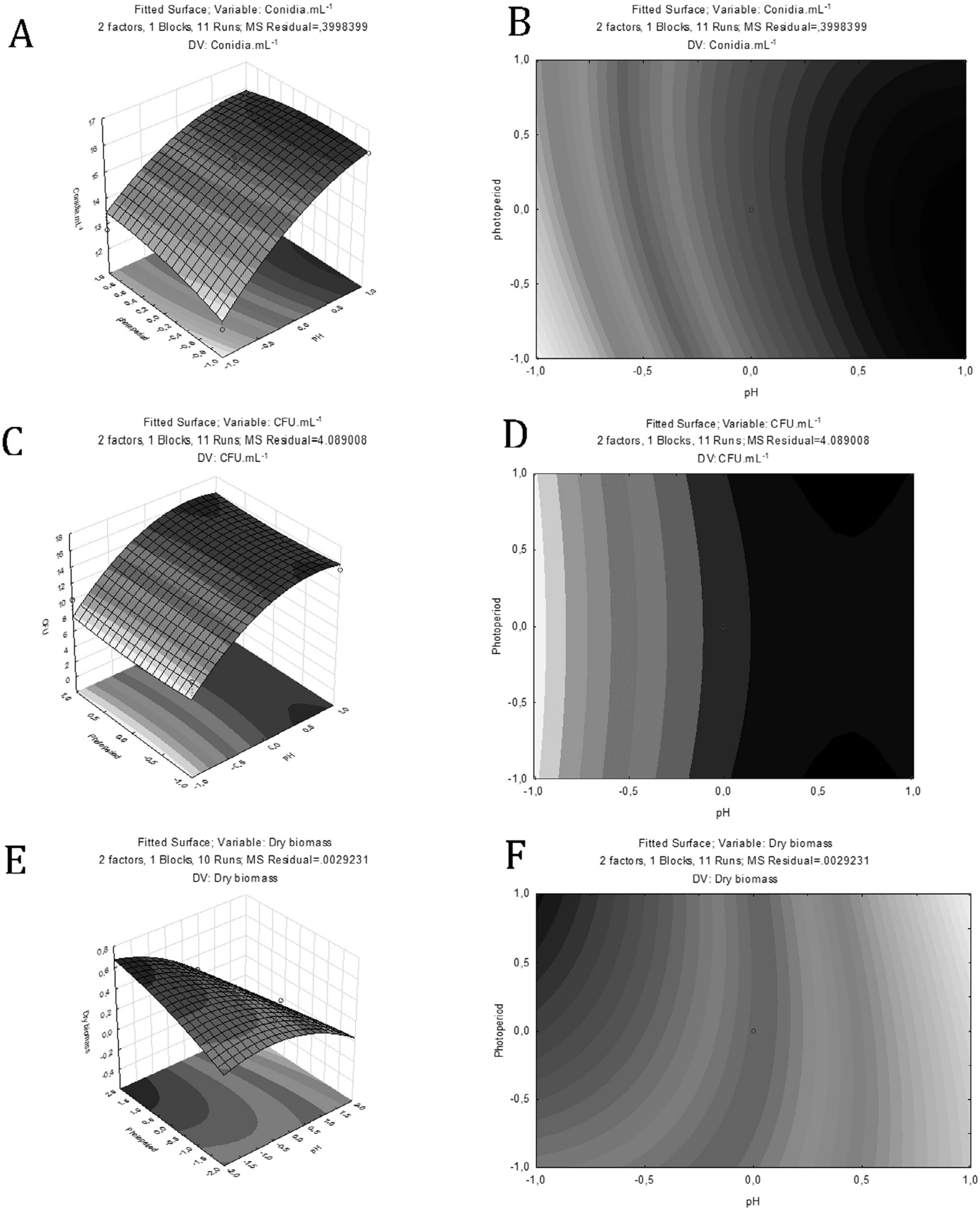

Fig. 3. Contour surfaces generated by the proposed model. Concentrations of conidia mL^{-1} (AB), CFUs mL^{-1} (CD) and dry biomass (EF) of *Clonostachys rosea* in optimized culture medium.

conidial production in the benchtop bioreactor, whereas an initial pH ranging from 2 to 2.3 had a very negative impact on conidial production in *C. rosea*. The pH was measured daily, and a value of pH 8.27 was observed after 7 days, which had the tendency to stabilize (Fig. 4). Sun et al. (2013) observed that the highest *C. rosea* conidial production was at the initial pH, which was close to neutral. This may explain why the pH was basic at the end of the fermentation process in the bioreactor. In addition to the pH, the photoperiod had a significant effect on conidial production (Tables 2 and 4). According to Sutton et al. (1997), light is important for the sporulation of this fungus, which is severely reduced

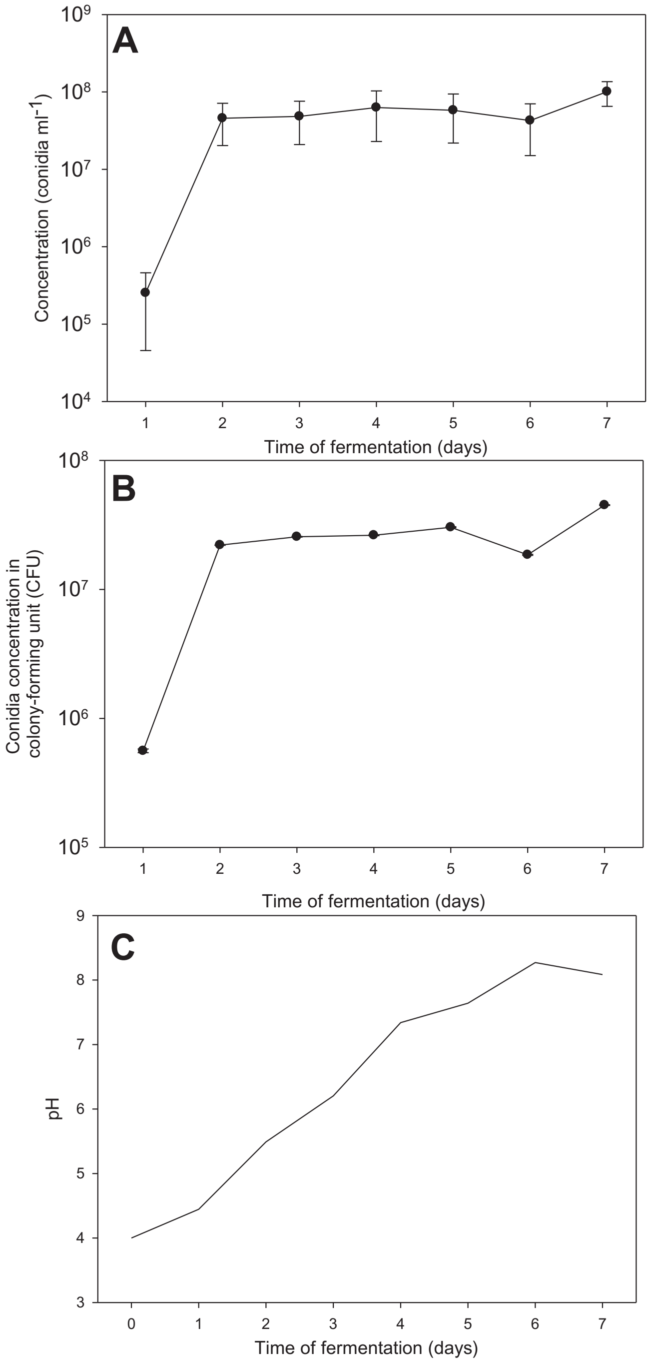

Fig. 4. Conidial concentration of *Clonostachys rosea* (LQC 62) produced in a benchtop bioreactor. A) Evaluation of the conidial concentration. B) Evaluation of the colony-forming units (CFUs) mL^{-1}. C) Evaluation of the medium pH by the time to fermentation (days). Error bars show the standard deviation of three replicates.

in compete darkness. In the present study, the highest conidial production was observed with 12 h of light. It is reported that the duration of light, but not its intensity, enhances *C. rosea* sporulation on leaf tissues (Shafia et al., 2001), a feature that might be related to the ecological lifestyle of the fungus, which is often associated with plant shoots and therefore exposure to light. Thus, providing light throughout the fermentation process is recommended for a higher yield of conidia. At the industrial scale, however, light intensity should be assessed for economic feasibility.

Based on our results, the composition of the culture medium and the type of *C. rosea* strain influence sporulation capability in different manners. In contrast to our results, Sun et al. (2013) showed the highest conidial production in submerged cultures at optimal initial pH values from 6.0 to 6.5. Additionally, the temperature of the culture from 27 to 30 °C increased *C. rosea* sporulation. These authors indicated that the maintenance of a low pH value during the fermentation process induced chlamydospore formation rather than conidial formation.

A significant advantage of LSF is a decrease in the time required for the mass production of *C. rosea* conidia. In the present work, the use of laboratory benchtop bioreactors with optimized culture medium and conditions increased the concentration of conidia in the first 2 days, remaining stable until the 6th day and increasing to 1×10^8 conidia mL^{-1} on the 7th day (Fig. 4A). For comparison, SSF employing rice or wheat grains as a substrate requires approximately 15 days to produce 5×10^8 conidia g^{-1} (Morandi, unpublished data). It is important to note that production of conidia using LSF requires much less space and labor, which is a crucial consideration with regard to the energy and labor costs for mass production.

Of note, traditionally available protocols for *C. rosea* solid-state fermentation require at least 2 weeks to achieve the same concentration (Viccini et al., 2007). We have shown that this is possible using liquid fermentation for a total of 6 days (Fig. 4A). When utilizing laboratory benchtop bioreactors with optimized culture medium and conditions, the increase in the conidial concentration is already noticeable on the 2nd day (Fig. 4A) and increases at steady rate until the 6th day (Fig. 4). Therefore, not only is there an associated reduced labor cost when adopting liquid fermentation but also a lower amount of time necessary to achieve a similar concentration (at least a 50% reduction), indicating that a higher inoculum can be produced within the same time frame.

A reduction in the time to spore production is observed when comparing submerged media with the solid-grain media. Our optimized culture medium reduced the process to between 48 h to 1 week (Fig. 4A), whereas solid media require weeks of fermentation. These results are very close to those observed by Kobori et al. (2015) with *T. harzianum*. For *Gliocladium virens* (Gl-3), no effect on the time required, between 3 and 15 days of fermentation, to ferment a given number of conidia in liquid medium was observed (Papavizas, 1985). These results agree with our data presented in Fig. 4.

An increase in conidial production 2 days after inoculation, with no subsequent significant increase, was observed in our bioreactor experiments, a time when the pH reached 5.2 and the culture medium was subsequently and progressively buffered to the point of alkalinization. Therefore, culture broth alkalinization was a consistent parameter that promoted *C. rosea* conidiogenesis.

When utilizing a bioreactor, it is possible to control the temperature, pH, agitation, pressure, dissolved oxygen and aeration throughout the fermentation process. Jakubíková et al. (2006) reported fermenter aeration to be a critical factor in conidial production, observing the need for oxygen supplementation to maintain required levels. However, pH and aeration were not controllable in the tests carried out in the present study, and analysis of aeration was performed by means of the culture medium to volume ratio in Erlenmeyer flasks (2.5: 1). In the tests to validate results in the bioreactor, different values were observed in preliminary tests; the pH of the samples was measured daily, and after 7 days, a value of pH 8.27 was observed. This value tended to stabilize but was well above the adjusted initial value (pH = 4.0). Daily pH assessment showed that the culture medium tended to alkalinize, consequently increasing the concentration of conidia, which agreed with the data observed in the CCD. Although the initial pH was not important for *Trichoderma* spp. conidia and chlamydospore formation (Lewis and Papavizas, 1983), other reports have shown better growth and conidiation with *Trichoderma* spp. in liquid media at pH 4 (acidic) than at any other value (Brown and Holsted, 1975). For many organisms, the standard pH range for optimal growth is 5 to 7.

The efficacy of the conidia obtained through liquid fermentation in disease control is crucial and is one of the problems in the production of biocontrol agents (Jaronski, 2014). In our study, germination of the conidia produced in LSF was > 90%, and their ability to colonize

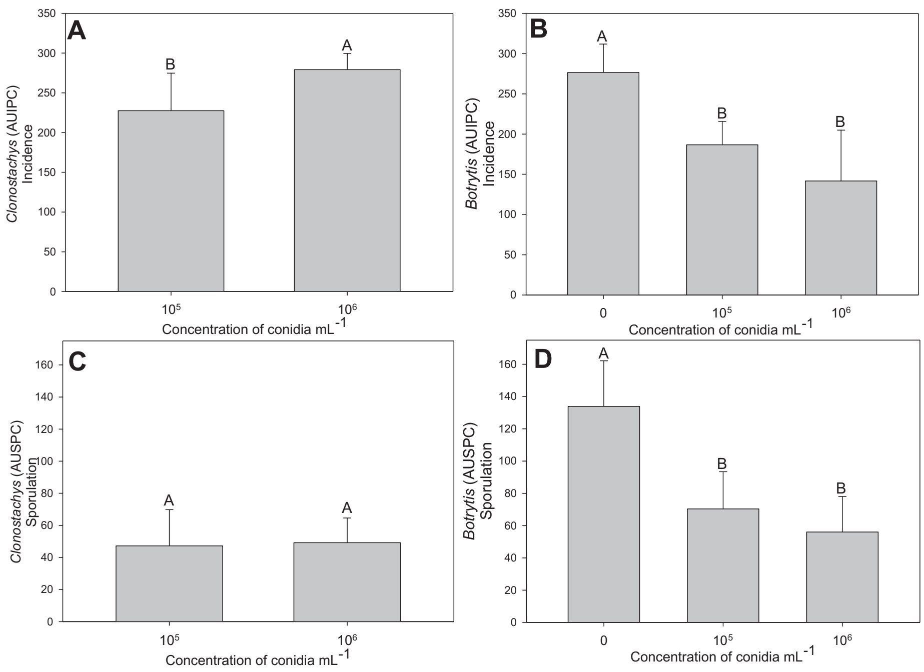

Fig. 5. Incidence (AC) and sporulation intensity (BD) of *Clonostachys rosea* and *Botrytis cinerea*, applied isolated or in combination, to strawberry leaf discs. A) Area under the incidence progress curve (AUIPC) of *C. rosea*. B) AUIPC of *B. cinerea*. C) Area under the sporulation progress curve (AUSPC) of *C. rosea*. D) AUSPC of *B. cinerea*. Data show the AUIPC or AUSPC ± standard deviation of three independent experiments. *C. rosea* was applied at 10^5 or 10^6 conidia mL^{-1} and *B. cinerea* at 10^5 conidia mL^{-1}. Both fungi were applied alone or in combination onto the same leaf disc. Small letters compare averages in rows, and capital letters compare averages in columns. Averages with the same letters are not significantly different (Tukey test, $p < .05$).

strawberry leaf discs as well as their ability to suppress *B. cinerea* sporulation was not affected (Fig. 5). These results are similar to those of Costa et al. (2016) using the same strain and conidia produced in agar medium, as well as those obtained by Morandi et al. (2000) with conidia obtained using rice grains.

Low-cost, large-scale production is necessary for the agricultural market, and production using grain suffers from high labor costs, long fermentation times, poor quality control, environmental concerns for workers and difficulties in scaling-up (Kobori et al., 2015). Indeed, a short process time is extremely important because it reduces labor and energy costs. For example, rice-based *C. rosea* fermentation requires 2 weeks for production and yields 5×10^8 conidia g^{-1}. However, Zhang et al. (2015) recently reported production of 3.5×10^{10} conidia g^{-1} of dry matter in 11 days using a mixture of wheat bran and maize meal (3:1) solid medium in a novel reactor that is light transparent and ventilated. In optimized liquid medium, we obtained concentrations of 4.50×10^7 conidia mL^{-1} (Fig. 4A) and 2.50×10^7 CFUs within 48 h of cultivation (Fig. 4B). According to a survey conducted by Brazilian biological control companies, the cost to ferment 1 kg of rice grains for mass production of *Trichoderma* sp. is approximately $7.07 to $14.67 USD, depending upon the final concentration (10^8 CFUs g^{-1} of *Trichoderma*). For the same two companies using submerged fermentation, the cost would drop to $3.08 to $ 6.62 USD per L (10^8 CFUs g^{-1} of *Trichoderma*).

One of the reasons that bacterial-based biocontrol is more competitive than fungal-based biocontrol is not due to the higher efficacy but rather to the lower cost. One of the reasons for this higher cost of fungal-based biocontrol is because it mostly relies on solid-state fermentation. By examining the carbon source (sucrose at 10 g L^{-1}), C:N ratio (200:1), temperature (25 °C), pH (3.555) and light duration (12 h) and performing validation in scaled-up bioreactor trials and evaluation of viability and disease control effectiveness, we propose that a gradual shift in the industry to liquid-based fermentation is feasible and that a more cost-effective *C. rosea*-based product is possible. In this way, the industry will be able to mass produce conidia in a rapid manner, which will eventually allow for the timely generation of products to control gray mold outbreaks.

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