Crop optimization and pre-steps standardization to get a *Bipolaris euphorbiae*-based bioherbicide[1]

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RESUMO

Otimização do cultivo e padronização de pré-etapas para a obtenção de bioherbicida à base de *Bipolaris euphorbiae*

Disponibilizar bioprodutos para o mercado implica em buscar processos adequados para a produção massal e formulação de agentes biológicos. Neste trabalho, foi examinada a produção de *Bipolaris euphorbiae* em meio sólido (fermentação em substrato sólido) e em sistema bifásico (cultivo em meio líquido seguido de cultivo em meio sólido), e determinados os processos de secagem e coleta de conídios, em condições de laboratório. Investigou-se a influência do período de incubação e a quantidade de inóculo. A secagem dos conídios foi realizada por meio de estufa (30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC e 60ºC) e fluxo laminar, fluxo contínuo de ar e câmara asséptica em temperatura ambiente. Os conídios secos foram obtidos por peneiramento e moagem em moinho de esfera, de martelo ou de grãos. Avaliou-se a viabilidade dos conídios e a esporulação no meio sólido e sistema bifásico. Considerando-se o período de crescimento, a melhor esporulação no meio sólido foi obtida após 10 dias de incubação, alcançando 8,3 x 10^7 conídios g^{-1} de substrato. O sistema bifásico não incrementou a esporulação de *B. euphorbiae* (4,5 x 10^7 conídios g^{-1} de substrato) cultivado por 14 dias, e a quantidade de inóculo líquido usado neste sistema não foi determinante para incrementar a sua produção. O fluxo contínuo de ar e o fluxo laminar mantiveram a viabilidade dos conídios (94,6% e 99,1%, respectivamente) e promoveram grande perda de umidade (62,6% e 54,0%, respectivamente). Todos os processos de moagem reduziram a germinação dos conídios (86,2%, 10,5% e 12%, respectivamente), enquanto o peneiramento proporcionou a coleta de conídios em pó com alta viabilidade (94,8%).

PALAVRAS-CHAVE: Produção massal; bioprodutos; coleta de conídios.

ABSTRACT

Making bioproducts available to the market requires finding appropriate processes for mass production and formulation of biological agents. This study aimed at evaluating the *Bipolaris euphorbiae* production in a solid medium (fermentation in solid substrate) and in a biphasic system (growth in a liquid medium followed by growth in a solid medium), as well as determining the processes for collecting and drying conidia, under laboratory conditions. The influence of the incubation period and inoculum quantity were also investigated. The conidia were dried by using an oven (30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC and 60ºC), and laminar flow, continuous air flow and aseptic chamber at room temperature. Dry conidia were obtained by sieving and grinding in a ball mill, hammer mill or grain grinder. The conidia viability and sporulation efficiency were evaluated in the solid medium and in the biphasic system. For growth period, the best sporulation on solid medium was obtained after 10 days of incubation, reaching 8.3 x 10^7 conidia g^{-1} of substrate. The biphasic system did not increase the *B. euphorbiae* sporulation (4.5 x 10^7 conidia g^{-1} of substrate), after 14 days, and the amount of liquid inoculum used in this system was not an important factor for increasing its production. The continuous air flow and laminar flow preserved the conidial viability (94.6% and 99.1%, respectively), while promoting a great moisture loss (62.6% and 54.0%, respectively). All the grinding processes reduced the conidia germination (86.2%, 10.5% and 12%, respectively), while sieving allowed the collecting of powdered conidia with high viability (94.8%).

KEY-WORDS: Mass production; bioproducts; conidia collecting.

INTRODUCTION

In agricultural environments, weeds compete directly and indirectly with economically important crops, reducing their yield, interfering with harvesting operations and acting as alternative hosts for insect pests and pathogens, in addition to affecting the quality of the harvested product (Barreto 2009). The weeds interference is responsible for reducing the agricultural production in 30-40% in the tropics (Lorenzi 2000), and it represents the largest barrier to food production, in many regions of the world (Muzik 1970).

Euphorbia heterophylla L. (Euphorbiaceae), popularly known as milkweed or wild poinsettia, is

one of the weeds most feared by soybean producers, because of the damage it can cause and the difficulty for controlling it (Brighenti & Oliveira 2011). Currently, the *E. heterophylla* control is mainly performed with the aid of herbicides that inhibit the enzymes acetolactate synthase (ALS) and protoporphyrinogen oxidase (PROTOX) (Vidal & Merotto Júnior 2001).

The recurrent use of certain herbicides, or herbicides with similar mechanisms of action, in the same area has led to the selection of weed populations that are resistant to certain chemical groups and that become difficult to control (Oliveira Júnior & Inoue 2011). For *E. heterophylla*, biotypes resistant to ALS inhibitors, or with multiple resistances to ALS and PROTOX inhibitors (Trezzi et al. 2005) and glyphosate (Vidal et al. 2007), have been identified. Moreover, environmental problems, such as contamination of food, soil and water, biological imbalances and toxicity to farmers, are among the additional consequences of the intensive and indiscriminate use of chemical pesticides. Such situations make the use of biological agents for weed control particularly attractive (Fontes 1992), following a global tendency towards seeking alternative control systems that are simultaneously effective, economic and less harmful to the environment.

The use of bioherbicides represents a strategic component of integrated weed management programs, and the *Bipolaris euphorbiae* Muschovej & Carvalho fungus is a potential agent for the biological control of *E. heterophylla*. This fungus may cause, under ideal conditions, plant diseases that result in severe defoliation, stem damage and death of the host (Barreto & Evans 1998). However, there are still limitations that prevent the use of bioproducts in the field, particularly with respect to large scale production and methods of product storage. Thus, it is essential to develop formulations that overcome such limitations (Almeida et al. 2007).

Mass production at low cost and under controlled conditions is an important step towards ensuring that large quantities of the pathogen can be obtained (Moino Júnior 2000, Lima et al. 2010). In order to enable this production, Penariol et al. (2008) investigated liquid and solid media obtained from grains, grain derivatives and agro-industry wastes. The combination of these substrates were also evaluated for increasing the production of the fungus in a solid medium and in a biphasic culture system (Moraes 2009). However, the biphasic system did not increase its production. Moraes (2009) suggests that this result may be a consequence of an inadequate incubation period, or an insufficient amount of liquid inoculum used to seed the solid medium. Nevertheless, these cultivation aspects need to be investigated.

In the bioproduct production process, drying and obtaining infective units constitute important preliminary stages of the product formulation. It is extremely important to maintain the pathogen in infectious and dormant stages that are safe and easy to use. The keys to the prolonged pathogen survival are preventing germination and reducing metabolism as much as possible (Horaczek & Viernstein 2004). For this purpose, water extraction is necessary, so that the conidia keep a moisture content of 5-10%, and then can be extracted from the substrate (Silva & Mello 2007).

Given the need for more studies focused on the various stages of bioproduct manufacturing, the present study aimed to optimise the conditions for the mass production of *B. euphorbiae*, on a solid medium or biphasic system, and to identify appropriate processes for drying and collecting conidia, while preserving their viability.

MATERIAL AND METHODS

The isolate FCAV #569 of *B. euphorbiae* used in this study was maintained in a stock culture, in laboratory. For being used in the assays, the fungus was cultured on plates containing minimal Pontecorvo medium ($NaNO_3$ = 6.0 g; KH_2PO_4 = 1.52 g; $MgSO_4$ $7H_2O$ = 0.52g; KCl = 0.52 g; $FeSO_4$ = 0.01 g; $ZnSO_4$ = 0.01 g; glucose = 10 g; agar = 15 g; distilled water = 1,000 mL) (Pontecorvo et al. 1953), modified by Penariol et al. (2008), with the addition of peptone (2.0 g L^{-1}) and the substitution of glucose by starch (10 g L^{-1}). The fungus was grown in an incubator, for 10 days, at 25ºC ± 0.5ºC, with a 12-hour photoperiod provided by four fluorescent lamps of 20 W. The assays were conducted from January to December 2011, in Jaboticabal, São Paulo State, Brazil.

The substrates were used in a 60:40 proportion, based on dry weight (g g^{-1}). To prepare them, 30 g of soybean hulls were soaked in distilled water, at a 3:1 ratio (v w^{-1}), for 15 minutes, and then strained to remove the water excess, and 20 g of sorghum grain

were cooked in heated distilled water, at a 3:1 ratio (v w^{-1}), for 5 minutes, in low heat. The substrates were mixed and placed in 250 mL Erlenmeyer flasks. The medium was autoclaved at 121ºC and 1 kgf cm^{-2}, for 40 minutes. The cultures were inoculated by using a nickel-chrome needle to transfer three 8 mm diameter disks taken from colonies grown on modified minimal Pontecorvo medium. The flasks were maintained in an incubator, at 25ºC ± 0.5ºC, for 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 days, with a photoperiod of 12 hours.

For this assay, growth in a liquid medium was followed by growth in a solid medium. The liquid medium consisted of 8% of sugarcane molasses obtained from dilution in distilled water.

The liquid medium (100 mL) was placed in Erlenmeyer flasks and autoclaved at 121ºC and 1 kgf cm^{-2}, for 20 minutes. The fungus was inoculated as described above, and incubated at 25ºC ± 0.5ºC, for 3, 4, 5, 6, 7, 8, 9 and 10 days, with a photoperiod of 12 hours. After the specified period, the mycelial mass that had formed was ground in a SB 40 Black & Decker® vertical mixer, for 3 seconds, and then strained in a sieve with 1 mm mesh, resulting in a suspension of conidia and mycelium fragments. This suspension was used to inoculate the solid medium at 3 mL per flask (Machado et al. 2010). The solid medium was prepared by mixing soybean hull and sorghum grain, and placed in 250 mL Erlenmeyer flasks, as previously described. The flasks were maintained at 25ºC ± 0.5ºC, for 5, 6, 7, 8, 9 and 10 days, with a photoperiod of 12 hours.

Conidia yield and viability were evaluated at the end of the assay with only solid medium and of the assay with the biphasic cultivation. Samples with 1 g of solid substrate + fungus were transfered to tubes containing 9 mL of Tween 80® solution (0.1% v v^{-1}). The tubes were vigorously shaken, and the obtained suspensions were used to determine the number of conidia produced, with the aid of a Neubauer chamber. The yield and viability of conidia formed in the liquid medium were also determined by collecting 1 mL of the suspension used to seed the solid medium.

The conidial viability was determined as described by Francisco et al. (2006). Sterile microscope slides were covered with a thin layer of modified minimal Pontecorvo medium. In three regions of the culture medium, corresponding to areas previously marked in the slide, one drop of the designated fungus suspension was inoculated. The

slides were kept in an incubator, at 25ºC ± 0.5ºC, and, after 7 hours, 150 conidia per region were observed under an optical microscope, to determine the germinated or not germinated ones, in order to obtain the percentage of viable conidia. Three replicate slides were used for each suspension.

Aiming to increase the yield of this production method, suspensions were obtained from the liquid medium fungal culture, as previously described. Different suspension volumes (1 mL, 3 mL, 5 mL, 7 mL and 9 mL) were inoculated in the solid medium. The flasks were maintained at 25ºC ± 0.5ºC, with a photoperiod of 12 hours. The conidia yield and viability were evaluated as already described, after specific incubation periods, in the biphasic culture system.

The solid medium containing the cultured fungus was subjected to different conditions, for drying to a constant mass, while attempting to maintain conidial integrity. The following conditions were evaluated: a) oven temperatures (30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC and 60ºC); b) laminar flow at room temperature; c) aseptic chamber at room temperature; d) continuous air flow at room temperature (pressure of 3.5 ± 0.3 kgf cm^{-2} and flow rate of 15 L min.$^{-1}$). In all conditions (except for the "d" condition, which used a container connected to the drying equipment), the material was arranged on plastic trays previously disinfected with ethanol 96º GL. The room temperature ranged from 24ºC to 27ºC, during the drying procedure.

The conidia viability, before and after the drying process, was evaluated, as well as the degree of water loss. For the latter parameter, a relationship (expressed as percentage) was established between the initial and final weight of the material evaluated. To assess the viability before and after drying, suspensions containing 1 g of solid medium + fungus and 9 mL of Tween 80® solution (0.1% v v^{-1}) were prepared, and the test was performed according to the method described by Francisco et al. (2006).

The medium containing fungus, cultured and dried according to the methodology described above, was subjected to three grinding processes and to simple sieving, in order to obtain the conidia. The nature of the solid substrates used to produce the fungus allowed reducing these materials to a fine powder containing conidia (this reduction was not required for sieving).

The following equipments were used to obtain a fine powder: a) a ball mill (Marconi, model

MA-350) with closed chamber; b) a hammer mill (Metalúrgica Roma, model MR-320) containing four hammers and a 1 mm sieve; c) a grain grinder (Arbel, model MCF-55). To extract the conidia by sieving, a sieve with 1 mm mesh was closed and shaken manually, for 10 minutes. The material obtained after this treatment was then passed through a sieve with 0.25 mm mesh, in order to remove impurities. In the ball mill method, the material was added to a grinding bowl and subjected to intermittent beats, for 1 minute. In the grain grinder, the regulator bushing was adjusted, so that the grinders would reduce the material to a fine powder. Grinding was performed twice, to achieve a fine powder.

The conidial viability was evaluated before and after each of the conidia collecting methods tested. Suspensions were made from a sample (1 g) of dried material, before grinding, or from 1 g of powder obtained after grinding. The samples were then transferred to test tubes containing 9 mL of Tween 80® solution (0.1% v v^{-1}). For the conidia extracted by sieving, 0.1 g of material diluted in 9.9 mL of Tween 80® solution (0.1% v v^{-1}) was used. The conidia viability was determined as described by Francisco et al. (2006).

Statistical analysis was conducted with the aid of the ESTAT (1997) software, version 2.0. The experimental design was completely randomized (CRD) and the data (four replicates per treatment) were subjected to analysis of variance, by using the F test, and means were compared by the Tukey's test, at 5%. For conidia drying and collecting assays, a factorial design was used, with two factors: drying methods *versus* evaluation point (before and after drying), and collecting methods *versus* evaluation point (before and after collecting).

RESULTS AND DISCUSSION

Independently of whether the *B. euphorbiae* was grown on solid medium or in the liquid and solid phases of the biphasic system, the incubation time exerted no influence on the viability of the conidia produced (p ≥ 0.05), and germination efficiency remained above 97%, for all treatments (Tables 1 and 2).

For cultivation on solid medium, the fungus sporulation, after 14 days of culture, showed the greatest numerical value for this parameter (8.9 x 10^7 conidia g^{-1} of substrate), but was significantly different

Table 1. Conidial production and viability of *Bipolaris euphorbiae* on solid medium, at different incubation periods (Jaboticabal, São Paulo State, Brazil, 2011).

Incubation period	Sporulation	Viability
days	x 10^7 conidia g^{-1}	%
5	1.7 B	97.5 A
6	2.4 AB	98.3 A
7	4.7 AB	97.9 A
8	5.4 AB	99.4 A
9	6.7 AB	99.1 A
10	8.3 AB	98.8 A
11	7.3 AB	99.5 A
12	7.0 AB	99.5 A
13	8.0 AB	99.4 A
14	8.9 A	99.4 A
F test	2.52*	2.91*
C.V. (%)	31.09	0.89

Means with the same letters in the columns do not differ from each other by the Tukey's test (p ≤ 0.05). Original values, but statistical analysis performed with transformed data to log (x+1) and arcsine √x/100, respectively for sporulation and viability. * Significant at 5%.

only when compared to the sporulation obtained after 5 days of incubation (1.7 x 10^7 conidia g^{-1} of substrate) (Table 1).

Aiming at fungus mass production and obtaining large conidia quantities, the incubation for 10 days resulted in the most optimal "sporulation with high viability *versus* incubation period" relationship, although not significantly different from most the other incubation periods. Considering these data (Table 1), a 10-days incubation period would theoretically allow 3 production cycles, within a 30-days period, and would reach 24.9 x 10^7 conidia g^{-1} of substrate. For an incubation period of 14 days, which provided the greatest numerical value for sporulation in this assay, 2 cycles would be completed within 30 days, finally yielding 17.8 x 10^7 conidia g^{-1} of substrate. The mean daily sporulation achieved in the first hypothetical situation would be 32% higher, suggesting that a 10-days incubation period would be optimal for producing the fungus.

The sporulation rate obtained with a 10-days incubation period (8.3 x 10^7 conidia g^{-1} of substrate) was higher than that observed by Moraes (2009): 2.3 x 10^7 conidia g^{-1} of substrate, under the same conditions. This difference is likely due to variations in the substrate quality of the solid media, as they were made at different times.

Production systems using submerged liquid culture are widely used for the mass production

Table 2. Conidial production and viability of *Bipolaris euphorbiae* on liquid and solid medium, at different incubation periods (Jaboticabal, São Paulo State, Brazil, 2011).

Incubation period	Liquid medium		Solid medium[1]	
	Sporulation	Viability	Sporulation	Viability
days	x 10^5 conidia mL^{-1}	%	x 10^5 conidia mL^{-1}	%
3	1.9 B	99.5 A	-	-
4	4.0 AB	99.7 A	-	-
5	3.8 AB	99.8 A	1.0 B	98.9 A
6	5.1 AB	99.8 A	2.2 AB	99.2 A
7	6.1 A	99.9 A	4.5 A	98.7 A
8	4.8 AB	99.7 A	4.0 A	98.7 A
9	5.1 AB	99.4 A	3.2 A	98.9 A
10	5.0 AB	99.3 A	3.7 A	98.8 A
F test	2.44*	3.1*	5.86**	2.63^{ns}
C.V. (%)	30.23	0.24	24.3	0.24

[1] Inoculum obtained after 7 days of incubation in liquid medium. Original values, but statistical analysis performed with transformed data to log (x+1) and arcsine √x/100, respectively for sporulation and viability. ^{ns} Non significant. ** and *: Significant at 1% and 5%, respectively.

of bioherbicides (Churchill 1982, Stowell 1991), however, with *B. euphorbiae*, sporulation in liquid medium without agitation and with different incubation periods yielded 10^5 conidia mL^{-1} (Table 2). Based on the data analysis, and considering that the fungus produced in this liquid phase was used as the inoculum for the second phase of the biphasic culture system, an incubation period of 7 days was chosen because it had the greatest observed numerical value (6.1 x 10^5 conidia mL^{-1}) for fungal sporulation, despite not statistically different from most other values. In culture on solid medium, incubation for the same period also provided the greatest numerical sporulation value, although not significantly different (p ≥ 0.05) from any other tested growth period, except for 3 days (Table 2). Data analysis suggests that the production of *B. euphorbiae* in the biphasic culture system would optimally require a total of 14 days, whereas Moraes (2009) used 17 days.

Sporulation was significantly higher when 7 mL or 9 mL of fungal suspension was used to seed the solid phase (Table 3). However, the amount of inoculum may not be responsible for the greater sporulation of the fungus in the biphasic system. The conidia production, even with larger inoculum, was 28.9% lower than that obtained with the cultivation only on solid medium (Table 1). In addition, seeding the substrate in three separate flasks, with 3 mL inoculum each, would theoretically make it possible to obtain a total production of 8.7 x 10^7 conidia g^{-1} of substrate, which is greater than that obtained by seeding the substrate in a single flask with 9 mL inoculum (5.9 x 10^7 conidia g^{-1} of substrate)

Table 3. Conidial production and viability of *Bipolaris euphorbiae* in the biphasic system, after inoculating the solid phase with different amounts of inoculum from culture in the liquid phase (Jaboticabal, São Paulo State, Brazil, 2011).

Inoculum quantity	Sporulation	Viability
mL	x 10^7 conidia g^{-1}	%
1	1.5 C	98.7 A
3	2.9 B	99.0 A
5	3.6 AB	98.9 A
7	4.1 AB	98.8 A
9	5.9 A	98.8 A
F test	13.17*	0.73^{ns}
C.V. (%)	13.7	0.91

Means with the same letters in the columns do not differ from each other by the Tukey's test (p ≤ 0.05). Original values, but statistical analysis performed with transformed data to log (x+1) and arcsine √x/100, respectively for sporulation and viability. * Significant at 5%. ^{ns} Non significant.

(Table 3). This is an industrially important aspect to consider.

Comparing the method of fungus production on solid medium and the biphasic system (Tables 1 and 2), the best sporulation *versus* incubation relationship was obtained by the cultivation on solid medium, which not only reduced the incubation time, but also provided a greater fungal sporulation. The process of removing 8 mm discs from plates containing the fungus may be less disruptive than grinding the mycelial mass in liquid medium, thus allowing faster and more efficient colonisation of the solid medium. Notably, the process of production on solid medium currently used in Brazil is simple and minimally automated, and yet it is efficient and cost compatible

with the national production structure (Morandi & Bettiol 2009).

The analysis of variance showed that the interaction of drying methods *versus* evaluation point was significant (F = 48.80**). The examination of this interaction (Table 4) allowed assessing the effect of drying methods on the conidia. The germination rate before drying remained above 98%, for all treatments, thereby ensuring sample homogeneity.

The most significant effects of the drying method on conidia were obtained for oven temperatures at 55ºC and 60ºC, which featured reductions in germination of 26.3% and 73.6%, respectively. For all the other methods, the viability of conidia was generally preserved after drying and remained above 94% (Table 4), a satisfactory level of quality for a commercial product.

The purpose of drying the material is to reduce the available water to a level capable of maintaining the viability of conidia for the longest storage period possible. The continuous air flow drying method provided the greatest loss of moisture content, but it did not differ significantly from the laminar flow method or the oven at 50ºC and 60ºC (Table 5).

Among the drying methods used in the present study, the continuous air flow and laminar flow can be considered the most suitable for drying *B. euphorbiae* conidia, because they provided a great moisture loss, while maintaining the conidia viability. The physical structure of the material used, containing large

Table 5. Moisture loss by material from fungal culture on solid medium, after different drying methods (Jaboticabal, São Paulo State, Brazil, 2011).

Drying method	Moisture content loss (%)
Laminar flow[1]	54.00 AB
Continuous air flow[1]	62.65 A
Aseptic chamber[1]	34.55 C
Oven at 30ºC	21.70 D
Oven at 35ºC	22.90 D
Oven at 40ºC	24.38 D
Oven at 45ºC	45.37 BC
Oven at 50ºC	50.66 AB
Oven at 55ºC	48.99 B
Oven at 60ºC	54.62 AB
F test	38.12**
C.V. (%)	5.62

[1] Room temperature. Original values, but statistical analysis performed with transformed data to log 5. ** Significant at 1%.

surface area and absence of aggregation, possibly facilitated the aeration by drying methods that did not use oven, allowing greater removal of water, which is important for the conidia preservation. Temperatures of 55-60ºC also provided great moisture loss, but caused a large reduction in the conidia viability (Tables 4 and 5). These temperatures may have altered the biochemical components of the metabolic machinery of the conidium, thereby negatively affecting its germination.

Tolerance to drying is a factor that may limit the success and trade of bioherbicides (Montazeri & Greaves 2002). The decrease in the metabolic activity of conidia reduces the viability loss during storage and minimises the production of toxic metabolites (Guijarro et al. 2006). Reports in literature suggest that the ideal moisture content for substrates containing conidia ranges from 10% to 30% (Silva & Mello 2007, Lomer & Lomer 1998).

The drying methods often used by the industry to process products based on microorganisms are freeze drying, spray drying and fluidised bed drying (Jin & Custis 2011). The differences between the technology and equipment used by these methods and those used in the present study greatly complicate the comparison of results. Nevertheless, the reduction observed in the *B. euphorbiae* viability, after drying at the highest temperatures, was also observed when other pathogens were dried by using other aforementioned common methods (Larena et al. 2003, Horaczek & Viernstein 2004), with subsequent irreversible damage to the biological materials, due

Table 4. Conidial viability of *Bipolaris euphorbiae*, regarding significance of results and considering the interaction drying methods *versus* evaluation point (Jaboticabal, São Paulo State, Brazil, 2011).

Drying method	Conidial viability (%)		F test
	Evaluation point		
	Before drying	After drying	
Laminar flow[1]	99.6 Aa	99.1 ABa	0.72[ns]
Continuous air flow[1]	98.9 Aa	94.6 Bb	9.13**
Aseptic chamber[1]	98.9 Aa	98.7 ABa	0.08[ns]
Oven at 30ºC	98.9 Aa	99.4 Aa	0.01[ns]
Oven at 35ºC	98.4 Aa	97.3 ABa	0.74[ns]
Oven at 40ºC	99.2 Aa	99.4 Aa	0.04[ns]
Oven at 45ºC	98.6 Aa	99.2 Aa	0.02[ns]
Oven at 50ºC	99.3 Aa	96.9 ABb	5.52*
Oven at 55ºC	98.9 Aa	72.9 Cb	97.82**
Oven at 60ºC	99.2 Aa	26.2 Db	473.65**
F test	0.39[ns]	98.56**	

[1] Room temperature. Original values, but statistical analysis performed with transformed data to arcsine $\sqrt{x}/100$. Means followed by the same capital letter in the column or small letter in the line do not differ from each other by the Tukey's test (p ≤ 0.05). [ns] Non significant. ** and *: Significant at 1% and 5%, respectively.

to excessive heat and water loss (Daemen & Van der Stege 1982). The drying methods used in the present study may not provide the ideal moisture content for substrates containing conidia, however, their simplicity can help to reduce the production cost of a bioherbicide based on *B. euphorbiae*.

The data analysis showed that the interaction of collecting methods *versus* evaluation point was significant, and that this interaction affected the conidia viability.

There was no significant difference in the germination of previously dried conidia, before they were subjected to the various collecting methods, showing that all treatments, initially, had viable particles (Table 6). Methods of collecting conidia by milling caused damage to the physical structure and integrity of conidia, as verified with microscope observation, thus significantly reducing their germination. This effect was especially pronounced when using the ball mill, which reduced germination by 86.2%. The aggressiveness of this method is due to the intermittent impact of a stainless steel ball against the milling chamber wall, which reduces the samples to a fine powder. The grain grinder and hammer mill reduced, respectively, 12.0% and 10.5% the conidia germination. Collecting conidia by sieving, although simple to perform, was the most appropriate method, allowing the extraction of high viability conidia (Table 6).

Studies performed to evaluate conidia extraction methods were not found in literature, fact that illustrates the need to investigate this preliminary stage in the process of formulating bioproducts. It is important to consider that the method for collecting

conidia must be compatible with the type of formulation desired. Irrespective of the formulation, the product should first be subjected to drying, to ensure that the conidia will remain viable during storage (Lomer & Lomer 1998).

CONCLUSIONS

1. The incubation period influenced the production of *B. euphorbiae* cultivated both on the solid medium and in the biphasic system, such that the optimal incubation period was 10 days for the solid medium and 14 days for the biphasic system.
2. The amount of liquid inoculum used to seed the solid phase did not increase the production of *B. euphorbiae* by the biphasic system, when compared to the cultivation only on solid medium.
3. The *B. euphorbiae* viability was influenced by the method for drying the fungus and collecting the conidia.

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Table 6. Conidial viability of *Bipolaris euphorbiae*, regarding significance of results and considering the interaction collecting methods *versus* evaluation point (Jaboticabal, São Paulo State, Brazil, 2011).

Collecting method	Conidial viability (%)		F test
	Evaluation point		
	Before collecting[1]	After collecting	
Grain grinder	88.92 Aa	78.33 Bb	8.2038**
Hammer mill	93.85 Aa	83.95 Bb	10.4640**
Ball mill	91.20 Aa	12.60 Cb	340.1616**
Sieving	94.87 Aa	94.82 Aa	0.0086ns
F test	2.0076ns	151.3616**	

[1] Drying the material containing the fungus under continuous air flow (room temperature). Original values, but statistical analysis performed with transformed data to arcsine $\sqrt{x/100}$. Means followed by the same capital letter in the column or small letter in the line do not differ from each other by the Tukey's test ($p \leq 0.05$). ns Non significant. ** Significant at 1%.

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