

SPORE PRODUCTION IN *PAECILOMYCES LILACINUS* (THOM.) SAMSON STRAINS ON AGRO-INDUSTRIAL RESIDUES

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ABSTRACT

Paecilomyces lilacinus has potential for pests control. We aimed to analyze mycelial growth and spore production in *P. lilacinus* strains in several agro-industrial residues and commercial media. This study suggests alternative nutrient sources for fungi production and that the biotechnological potential of agro-industrial refuses could be employed in byproducts development.

Key words: biological control, mycelial growth, nematophagous fungus.

Rational utilization of pathogens, whether from bacterial, fungal, nematode or arthropod origin, aiming pest maintenance at a non economic level with reduced environment aggression, has been adopted worldwide in biological control programs (1). The use of microbial agents for biological control of pests and plant diseases is an important strategy to minimize synthetic chemical pesticides effects in humans, animals and to the environment (21).

Fungi are the microorganisms that hold the highest potential for biological control (16), they have a limited host range, with minimal impact on non-target species (14). The current working hypothesis for the number of fungi on Earth was estimated in 1-to 5 million species (13). These estimated data provide potential searching material for finding new fungal agents that could be able to cause epizootics on insect and nematodes populations. These fungi also can be isolated as endophytes (26), living within host plants without causing any noticeable symptoms of disease (3,8,27). It is hypothesized that the endophytes, in contrast to known pathogens, generally have far greater phenotypic plasticity and thus more options to interact with their host than pathogens (29).

The genus *Paecilomyces*, which is widespread in nature, assembles several entomopathogenic species (1). *Paecilomyces*

lilacinus is a soil fungus with a good potential for biological control. This specie has been described as being as efficient as the commonly used nematicides (17,28) and also as a controller of greenhouse insects and mite pests (10).

Fungi can be manipulated in several ways for use in biocontrol, but must be available in large quantities (1). The success of microbial control, often depends upon the pathogen at competitive prices (20). Production processes for fungal biopesticides must be low-cost and yield high concentrations of viable, virulent, and persistent spores (14). The nutritional composition of the production medium has a significant impact on the attributes of the resulting propagules, such as biocontrol efficacy, desiccation, tolerance, and persistence (12,18).

A great alternative to achieve a satisfying price is the utilization of industrial residues or agricultural products. The agricultural wastes are produced in large quantities in many States in Brazil, including the Paraná State. The biotechnological potential of these refuses can be employed in byproducts development and improvement. Several studies have been performed on the utilization of agro-industrial residues with added value (23,31,33). Many materials have been tested for spore production by entomopathogenic fungi, such as sorghum, broad bean, beans, cassava bagasse, rye flour, cassava

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flour, rice and residues such as sugar-cane bagasse and refused potatoes (5,7,9,32).

Ayala (2) mentioned the utilization of refuse potato in the entomopathogenic fungus *Beauveria bassiana* large-scale production. Brand *et al.* (4) selected low-cost substrate for spore production of *P. lilacinus* in solid state fermentation.

In this paper we aimed to analyze the mycelial growth and spore production in *Paecilomyces lilacinus* strains in Refuse Potato natural medium and Potato Dextrose Agar (PDA) commercial medium. The strain that showed the highest spore production was evaluated in several agro-industrial residues and commercial media. The data available from these experiments could be employed in mass production of *P. lilacinus* for biological control.

The Brazilian strain Endo 69 was isolated as endophyte from soy plants by Pimentel (25). Mutant strains 2K and RG3, originally isolated from *Meloydogine incognita*, were obtained by U.V-light and GAMMA ray irradiation, respectively, by Pimentel and Azevedo (24). The fungal strains were maintained in the biological collection of Laboratório de Microbiologia e Biologia Molecular (LabMicro), Universidade Federal do Paraná, Curitiba, Paraná, Brazil. The substrate refuse potato was obtained from the local market (Curitiba). Cassava bagasse, rye, barley, wheat, soy and coffee husks substrates were gently donated by industrials from Paraná. The residues were dried at 55°C for 48 h, milled and sieved to obtain particles size between 0.8 and 2.0 mm. The flour production followed the protocol described by Ayala (2), with modifications in pH that was adjusted to 4.0. All natural media were added with 15% agar. The commercial media utilized were: Malt Agar (MA), Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) and were prepared following the manufacturer (Difco) instructions.

Paecilomyces lilacinus strains Endo 69, 2K and RG3 were grown on selected media for 7 days at 28°C in B.O.D. incubator. A sample of 0.5cm diameter was removed from the center of each colony and placed upside down in new plates. After incubation for 14 days, the colony diameter was recorded using 2 cardinal diameters. Spore production was estimated by removing a sample of 0.5cm diameter, tangentially from the inoculum. The spores were dispersed in Tween 80 (0.1%) suspension and counted in a Neubauer chamber. Seven replicate Petri dishes were prepared for each strain evaluated. The strain which showed the highest spore production rate was selected for further experiments as described below.

A 0.5ml aliquot of spore suspension from *P. lilacinus* selected strain in Tween 80 (0.1%) (10^8 spores.ml⁻¹) was placed in Erlenmeyer flasks containing 50 ml of PDA (maintained melted at 45°C) and mixed gently until the medium solidifies. This procedure was repeated 2 times. Then, the flasks were incubated at 28°C for 10 days.

The spore suspensions were prepared by addition of 40 ml sterile distilled water, 15 g of glass beads and Tween 80 (0.1%) at

the culture flask, and stirred for 30 minutes on a magnetic stirrer. The spores were counted in Neubauer chamber and the spore concentration was adjusted to 10^8 spores.ml⁻¹. Spores from *P. lilacinus* selected strain, obtained as described, were grown on selected agro-industrial residues media: Refuse Potato (RP), Cassava Bagasse (CB), Rye (R), Barley (B), Wheat (W), Soy (S), 80% Cassava Bagasse added with 20% Coffee Husks (CC), 80% Cassava Bagasse added with 20% Soy (CS80) and 90% Cassava Bagasse added with 10% Soy (CS90) and commercial media MA, SDA and PDA for 14 days at 28°C in B.O.D. Three replicate Petri dishes were prepared for each media evaluated data were analyzed by ANOVA, means were compared by a Tukey test at 1% or 5%, using software ASSISTAT version 7.5, 2008 (30).

Diameters from fungal colonies were evaluated after 14 days of grown. All strains assayed on Refuse Potato medium showed higher mycelial growth in contrast with PDA medium. The Endo 69 strain showed the highest mycelial growth only on PDA medium which differs statistically from 2K and RG3 strains ($p \leq 0.05$) (Table 1).

PDA and Refuse Potato media did not differ in promoting spore production in all strains assayed. However, the strain Endo69 showed the highest spore production rate, which is significantly different from strains 2K and RG3 ($p \leq 0.05$) (Table 1).

The *P. lilacinus* strain Endo 69 showed high rate of spore production on Refuse Potato medium and its efficiency is statistically comparable to commercial medium PDA and to the natural medium Cassava bagasse 80% and coffee husks 20% (CC). The media CS80 and CS90 composed by Cassava bagasse 80% and 90%, and soy 20% and 10%, respectively, as well Wheat (W) and Rye (R) media, showed no statistical difference ($p < 0.01$) (Table 2). The lowest spore production rate was observed in Soy medium, followed by Cassava bagasse and Barley media. The natural media evaluated did not show a performance as good as that displayed by Malt Agar commercial medium for spore production in *P. lilacinus* strain Endo 69. However, Refuse Potato medium provided spore production as efficiently as PDA medium (Table 2).

All *P. lilacinus* strains assayed showed the highest mycelial growth on RP medium in contrast with PDA commercial medium and no statistical difference was observed among them. However, on PDA, the mycelial growth of Endo 69 strain was higher than in 2K and RG3 strains.

The composition of natural Refuse Potato medium, which contains amino acids as lysine, methionine and cysteine, and several minerals, known as growth promoting factors, may have contributed to a higher mycelial growth in contrast with the observed in PDA commercial medium (34).

Although only a few studies had been performed in *P. lilacinus* in regards of mycelial growth in agro-industrial media, is well known that most fungi require nitrogen source as well as an utilizable carbohydrate for mycelial growth. Kamp and

Table 1. Mycelial growth and spore production in *P. lilacinus* strains Endo 69, 2K and RG3, after 14 days at 28°C, on PDA commercial and Refuse Potato natural media.

Strains	Culture media			
	Refuse-Potato		PDA	
	Colony diameter (mm) ⁽¹⁾	Spores.10 ⁸ .ml ⁻¹⁽²⁾	Colony diameter (mm) ⁽¹⁾	Spores.10 ⁸ .ml ⁻¹⁽²⁾
Endo69	56,4167 aA	1.5667 aA	51,8333 aB	1.6167 aA
2K	54,5000 aA	0.7833 bA	44,1667 bB	0.8250 bA
RG3	55.6677 aA	0.4583 bA	47,0833 bB	0.6417 bA

⁽¹⁾Means calculated from 3 replications. Data transformed to $\log(x+2)$. C.V.= 5.2901. ⁽²⁾Means calculated from 3 replications. Data transformed to $\log(x+2)$. C.V.= 37.1959. Means followed by the same small letter (strains) and capital letter (media) do not differ among them by Tukey test at 5%.

Table 2. Spore production in *P. lilacinus* strain Endo 69 on commercial and natural media after 14 days at 28°C.

Media	Spores.10 ⁸ . ml ⁻¹⁽¹⁾	
MA	2.6130	a
SDA	2.0806	b
RP	1.7546	c
PDA	1.6911	c
CC	1.5600	cd
CS90	1.3526	de
CS80	1.3307	de
W	1.1883	e
R	1.0770	ef
B	0.7959	fg
CB	0.6927	g
S	0.6780	g

⁽¹⁾Means calculated from 3 replications. Data transformed to $\log(x+1)$. C.V.= 7.55%. Means followed by the same letter do not differ among them by Tukey test at 1%.

MA: Malt Agar; SDA: Sabouraud Dextrose Agar, PDA: Potato Dextrose Agar, RP: Refuse Potato, CC: 80% Cassava Bagasse added with 20% Coffee Husks, R: Rye, B: Barley, CB: Cassava Bagasse, S: Soy, CS80: 80% Cassava Bagasse added with 20% Soy, CS90: 90% Cassava Bagasse added with 10% Soy, W: Wheat.

Bidochka (15) had demonstrated that growth in *B. bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* is affected by different solid substrate culture conditions as measured by colony diameter. Colony diameters on nutrient poor substrates were often similar to that of other media types but growth was extremely sparse. Ayala (2) assayed the potato refuse medium for spore production in *B. bassiana* and observed that this medium was more efficient when compared with PDA commercial medium.

Although PDA and RP media did not differ in promoting spore production in all *P. lilacinus* strains assayed, the strain Endo69 showed the highest spore production rate, which is significantly different from strains 2K and RG3. The low spore production rates observed in *P. lilacinus* mutant strains 2K and RG3, which were obtained by U.V-light and GAMMA ray irradiation, respectively, may be resultant of mutagenic agents effects. These mutational events could be responsible for altering genes involved in metabolic pathways that lead to spore production. Pacolla-Meirelles *et al.* (22) described two *B. bassiana* UV-light resistant mutants, which showed lower sporulation rates in contrast with wild strains.

High spore numbers is one of the main criteria for choosing a fungal pathogen for biological control of pests in the field. According to Martignoni (20), the success of microbial control, often depends upon the pathogen at competitive prices. Production processes for fungal biopesticides must be low-cost and yield high concentrations of viable, virulent, and persistent propagules (14). Through the knowledge that *P. lilacinus* strain Endo 69 showed higher spore production on RP medium, this strain was selected for further experiments on several agro-industrial media.

Once agricultural wastes are produced in large quantities in Brazil, this study provides a great contribution to employ these refuses with added value. Several studies have been performed on the utilization of agro-industrial residues, such as sugarcane and cassava bagasses, corn, wheat coffee husks, soy, glucose syrup, grape and beetroot syrup (6,23).

Methods for commercial production of conidia are usually done on solid substrates that can consist of cereal grains, rice or other starch-based substrate (11). According to Kamp and Bidochka (15), spore production in *B. bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* was affected by different solid substrate culture conditions. Different nutrient types in the agar media resulted in variability in the number of spores produced after the 14 days growth period. Sporulation likely

occurs upon nitrogen depletion in the presence of carbohydrate. For optimum sporulation a medium is required where extensive mycelial growth is followed by spore production. A nutrient rich medium would not stimulate sporulation while a nutrient poor medium would not offer extensive mycelial growth.

Leena *et al.* (19) evaluated mass production of *P. farinosus* and *P. lilacinus* on sugarcane molasses, spent wash and other agro-industrial wastes. Sugarcane pressmud supported the growth as well as significantly greater spore production of both species compared to other agro-industrial byproducts and wastes tested.

Among all the natural media assayed, RP allowed strain Endo 69 to produce higher spores number. Its efficiency for providing this particular capacity by the fungus was comparable to commercial medium PDA. Potato dextrose agar is usually applied for fungal cultures in laboratory conditions due its efficiency in providing mycelial growth and spore production. Upon the knowledge that agro-industrial refuse potato provides as well high spore production rates in *P. lilacinus* strain, which was similar to that observed on PDA, this natural media could be employed hereafter in mass production evaluations on this and others fungi with potential for biological control.

In regard of achieve low-cost and yield high concentrations of viable fungal spores and to make good use of agricultural wastes produced in large quantities in Brazil, this study suggests alternatives for nutrient sources aiming *P. lilacinus* mass production. The biotechnological potential of these agro-industrial refuses could be employed in byproducts development and improvement for biocontrol programs establishment.

RESUMO

Produção de esporos em linhagens de *Paecilomyces lilacinus* (Thom.) samson em resíduos agro-industriais

Paecilomyces lilacinus apresenta potencial para controle de pragas. O objetivo deste trabalho foi analisar o crescimento micelial e a produção de esporos de linhagens de *P. lilacinus* em resíduos agro-industriais e meios comerciais. Este estudo sugere fontes alternativas para produção de fungos com potencial biotecnológico para desenvolvimento de bioprodutos.

Palavras-chave: controle biológico, crescimento micelial, fungos nematófagos.

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