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Biotechnology of biomass production *in vitro* of fungi isolated from *Pinus*

Biotecnologia da produção de biomassa *in vitro* de fungos isolados de *Pinus*

Biotecnología de la producción de biomasa *in vitro* de hongos aislados de *Pinus*

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Abstract

Fungi are organisms capable of synthesizing metabolites of industrial interest and the standardization of biomass production for the extraction of these compounds has biotechnological applications. The objective of this work was to optimize the *in vitro* cultivation process for fungi isolated from *Pinus* sp., standardizing the best conditions for the production of biomass, contributing to its large scale production. Therefore, the conditions of *in vitro* cultivation of the fungi *Botrytis cinerea*, *Rhizoctonia* sp. and *Suillus* sp., were evaluated based on the maximum production of dry biomass (PBS), varying temperature, medium and cultivation time. The fungi were grown in glass flasks with liquid culture media, in a BOD chamber, without mechanical stirring. Potato-dextrose broth - PD broth (PD), Czapek - CZ broth (CZ) and Malt Extract - EM broth (EM) were evaluated at temperatures ranging from 8 to 32 ºC and incubation times from 7 to 35 days. PD broth showed better results for fungi *B.cinerea* and *Rhizoctonia* sp., when compared to CZ and EM broths, in PBS, while *Suillus* sp. showed better development in EM broth. The best growth temperature based on PBS was 12 ºC and 16 ºC, with 28 and 35 days of cultivation.

Ke**ywords:** *Botrytis cinerea*; *Rhizoctonia* sp.; *Suillus* sp.; Biotechnological optimization.

Resumo

Fungos são organismos capazes de sintetizar metabólitos de interesse industrial e a padronização da produção de biomassa para a extração desses compostos tem aplicações biotecnológicas. O objetivo deste trabalho foi otimizar o processo de cultivo *in vitro* para fungos isolados de *Pinus* sp., padronizando as melhores condições para a produção de biomassa, contribuindo para sua produção em larga escala. Portanto, as condições de cultivo *in vitro* dos fungos *Botrytis cinerea*, *Rhizoctonia* sp. *e Suillus* sp., foram avaliados com base na produção máxima de biomassa seca (PBS), variando temperatura, meio e tempo de cultivo.

Os fungos foram cultivados em frascos de vidro com meios de cultura líquidos, em câmara BOD, sem agitação mecânica. Caldos de batata-dextrose (BD), Czapek (CZ) e extrato de malte (EM) foram avaliados em temperaturas variando de 8 a 32 ºC e tempos de incubação de 7 a 35 dias. O caldo BD apresentou melhores resultados para os fungos *B. cinerea* e *Rhizoctonia* sp., quando comparado com os caldos CZ e EM, na PBS, enquanto que *Suillus* sp. apresentou melhor desenvolvimento em caldo EM. A melhor temperatura de crescimento com base no PBS foi de 12 ºC e 16 ºC, com 28 e 35 dias de cultivo.

Palavras-chave: *Botrytis cinerea*; *Rhizoctonia* sp.; *Suillus* sp.; Otimização biotecnológica.

Resumen

Los hongos son organismos capaces de sintetizar metabolitos de interés industrial y la estandarización de la producción de biomasa para la extracción de estos compuestos tiene aplicaciones biotecnológicas. El objetivo de este trabajo fue optimizar el proceso de cultivo *in vitro* de hongos aislados de *Pinus* sp., Estandarizando las mejores condiciones para la producción de biomasa, contribuyendo a su producción a gran escala. Por tanto, las condiciones de cultivo *in vitro* de los hongos *Botrytis cinerea*, *Rhizoctonia* sp. y *Suillus* sp., se evaluaron con base en la producción máxima de biomasa seca (PBS), variación de temperatura, medio y tiempo de cultivo. Los hongos se cultivaron en frascos de vidrio con medio de cultivo líquido, en una cámara de DBO, sin agitación mecánica. Se evaluaron caldos de papa-dextrosa (PD), Czapek (CZ) y extracto de malta (EM) a temperaturas que oscilan entre 8 y 32 ºC y tiempos de incubación de 7 a 35 días. El caldo PD mostró mejores resultados para los hongos *B. cinerea* y *Rhizoctonia* sp., En comparación con los caldos CZ y EM, en PBS, mientras que *Suillus* sp. mostró un mejor desarrollo en caldo EM. La mejor temperatura de crecimiento basada en PBS fue de 12 ºC y 16 ºC, con 28 y 35 días de cultivo.

Palabras clave: *Botrytis cinerea*; *Rhizoctonia* sp.; *Suillus* sp.; Optimización biotecnológica.

1. Introduction

Pinus is an exotic conifer that was brought to Brazil over a century ago. Experimentally, American species were introduced in 1948 due to their ease of cultivation. From the 1970s, plantations began to proliferate rapidly, in deforested areas, with high insolation, adapting to low fertility soils and resisting severe environmental conditions such as drought and frost (Shimizu, 2008). According to this author, the species of *Pinus taeda* L., *P. elliottii* Engel var. *elliottii*, *Pinus oocarpa* Schiede ex Schltdl. and *Pinus caribaea* Morelet are

examples. This plant has an important economic prominence and is used in the wood industry, in the production of cellulose, extraction of resin and production of coal (Brasil, 2018). Among the most recent applications of *Pinus*, studies of biomonitoring of the emission of pollutants in the environment by combustion stand out (Fernández-Varela, et al., 2015).

Fungi can parasitize pines, absorbing their nutrients and causing serious diseases, in seedlings and trees (Auer & Santos, 2016) or make beneficial associations with the plant (symbiosis), transferring nutrients, minerals and water from the soil to it, being able to protect the roots from the attack of pathogens (Moreira & Siqueira, 2006). Among the fungi that perform these interactions with *Pinus*, *Botrytis cinerea* Pers. (Fr.), *Rhizoctonia* sp. and *Suillus* sp.

Botrytis cinerea belongs to the Ascomycota division, order Helotiales, class Leotiomycetes and family Sclerotiniaceae (Mycobank, 2020). It is a pathogenic and necrotrophic fungus, causing the disease known as gray mold, and can be destructive in several fruit, vegetable and forest crops (Abuqamar, et al., 2017).

Rhizoctonia is a genus belonging to the division Basidiomycota, class Agaricomycetes, order Cantharellales, family Ceratobasidiaceae (Mycobank, 2020). They are filamentous fungi that live in the soil, usually associated with roots and can be phytopathogenic or saprophytic (Agrios, 2005), and even mycorrhizal (Moreira & Siqueira, 2006). Some species, such as *Rhizoctonia solani* Kuhn, cause diseases in pines, as rotting of cuttings and blight of seedlings (Agrios, 2005; Auer & Santos, 2016).

The genus *Suillus* belongs to the division Basidiomycota, class Agaricomycetes, order Boletales, family Suillaceae (Mycobank, 2020). They have about 50 species reported as mycorrhizal fungi. They are able to associate with roots of plants of the Pinaceae family forming ectomycorrhize (Verma & Reddy, 2015). These fungi play an important role in the attempt to reforest ecosystems as they are used as bioindicators of the quality of the environment (Sarwar & Khalid, 2014).

In addition to the fungus-plant interaction, it is known that fungi produce primary and secondary metabolites. Primary metabolites are produced during the vegetative growth phase of fungi and are essential for their growth and development. Secondary metabolites are produced when the microorganisms are in the stationary growth phase, synthesized from products or intermediate compounds generated by the primary metabolism, and which generally have biological action (Lopes, 2011). As examples of primary metabolites, we can mention the vast class of carbohydrates produced by fungi, especially polysaccharides, due to their industrial, technological and biological ablicability (Silva, et al., 2006). Recently, there

was the isolation and characterization of α-α-trehalose, a carbohydrate with cryoprotective activity, from the phytopathogenic basidiomycete *Bjerkandera adusta* (Willd.) P.Karst. (Rech, et al., 2020).

The chemistry and research of natural products from fungi is a field in constant growth, and microorganisms are sources of molecules of medicinal interest (Pinto, et al., 2002, Bérdy, 2005). Among fungi, the production capacity is more significant with ascomycetes, imperfect fungi, other filamentous and endophytic types (Bérdy, 2005). Some substances obtained can be mentioned as examples: penicillin from the fungus *Penicillium chrysogenum* Thom. discovered in 1928 by Alexander Fleming (Ligon, 2004) of great antibacterial potential. Querellou et al. (2010) in a review on fungi isolated from marine algae mentioned obtaining the polyketide asco-sali pyrrolidinone-A40, isolated from *Ascochyta salicorniae* with potential antimalarial activity; in addition to alkaloids with anticancer activity isolated from *Penicillium citrinum* Thom., *Fusarium sp., Apiospora montagnei* Sacc. According to Hasan et al. (2015), about 6,000 natural products were isolated from marine fungi, a promising source for obtaining antibiotics, antivirals, antifungals, anti-yeasts, anti-tumors and anti-inflammatories. These authors also reported the possibility of isolating chemical molecules from groups of secondary metabolites with recognized biological activity such as alkaloids, polyketides, terpenes, isoprenoid and non-isoprenoid compounds and quinones.

In vitro cultivation of microorganisms for the purpose of large-scale production and biotechnological application, requires basic information to optimize the conditions for their growth, such as culture medium, temperature, incubation time, pH, among others as shown by Hatvani et al. (2001). The culture media used for the growth of fungi can be liquid, solid or semi-solid and must have a source of carbon and nitrogen, in addition to mineral salts and growth factors (Rossi, et al., 2007). There is a wide variety of culture media, whose composition directly affects the growth characteristics of fungi, influencing the type, shape and productivity (Rossi, et al., 2007).

The objective of this work was to standardize *in vitro* culture conditions for fungi isolated from *Pinus*, describing the best conditions for biomass production, being a standardization contribution to a large scale production process.

2. Materials and Methods

The fungi of this study belong to the Collection of Fungos and Oomycetos Florestais, Embrapa Florestas, Colombo/PR, Brazil, preserved by Castellani method in small flasks with sterile water, at 4 ºC. Specimens of each fungus were deposited at the Herbarium of the University of Western Paraná - UNIOESTE under the numbers 4241 (*Botrytis cinerea*), 4243 (*Rhizoctonia* sp.) and 4245 (*Suillus* sp.).

Botrytis cinerea was isolated from diseased seedlings of *Pinus taeda* collected in Guarapuava / PR; *Rhizoctonia* sp. was isolated from sick seedlings of a hybrid of *Pinus caribaea* Morelet var. *caribaea x Pinus elliottii* Engelm var. *elliottii* collected in Itapeva / SP; *Suillus* sp. was isolated from basidioma in *P. elliottii* var. *elliottii* in Colombo / PR. *Botrytis cinerea* (= *Botryotinia fuckeliana* (from Bary) Whetzel) has a sequence deposited in the GenBank (KJ476441). The other fungi were not sequenced or deposited on GenBank.

Each isolate was grown in Petri dishes with potato-dextrose-agar medium - PDA (commercial extract of PDA, 39 g; ultra-purified water, 1000 mL) to produce inoculum in BOD chambers, at 24 ºC, in the dark, for 14 days. The fungi cultivation tests were performed in a liquid medium following the methodology of Silva et al. (2018), varying the type of broth, temperature and time of cultivation.

2.1 Culture medium test

Three liquid culture media were tested: potato-dextrose broth - PD broth, Czapek - CZ broth (sucrose 30 g; $NaNO_3$ 2 g; KH_2PO_4 1 g; $MgSO_4$ 0.5 g; KCl 1g; $FeSO_4$ 0.01 g; Ultra-purified water 1000 mL) and Malt Extract - EM broth. Two mycelium agar discs were removed from the pure culture of each fungi, in a laminar flow hood, and inoculated into glass flasks with a capacity of 500 mL sterilized in an autoclave containing 100 mL of broth. For each tested broth, ten flasks were incubated in a BOD chamber for 35 days, at rest and in the dark at a temperature of 12 ºC for the fungus *B. cinerea*, 16 ºC for *Rhizoctonia* sp. and *Suillus* sp. After 35 days of incubation, the fungal biomass was separated from the culture broth by filtration, using filter paper, and subjected to drying at 40 ºC for 72 h. Then, the production of dry biomass (PBS) was determined by gravimetry.

2.2 Cultivation temperature test

Mycelium agar discs (5 mm diameter) were removed from pure fungi cultures, and inoculated in glass flasks, with a perforated plastic lid and buffered with hydrophilic cotton and 500 mL capacity, sterilized in an autoclave (120 ° C, 1 atm, 20 min) containing 100 mL of Malt Extract - EM broth (20 g malt extract; 1000 mL ultrapurified water) for *Suillus* sp. and potato-dextrose broth - PD (Commercial potato and dextrose extract, 24 g; 1000 ml ultra-purified water) for *B. cinerea* and *Rhizoctonia* sp. Two mycelium agar discs were inoculated per flask, for each of the fungi and by temperature. For *B. cinerea*, temperatures 8, 12, 16, 20, 24, 28 and 32 °C were tested; for *Rhizoctonia* sp. and *Suillus* sp. 12, 16, 20, 24, 28 and 32 °C were tested. The flasks were incubated for 35 days in BOD chambers, at rest and in the dark. After this time, PBS is determined as in the previous assay.

2.3 Cultivation time test

Cultivation times 7, 14, 21, 28 and 35 days were tested in biomass production. The methodology for inoculation, cultivation and determination of PBS was the same as the previous tests. The flasks were incubated in BOD chambers at the best temperatures and culture medium for each fungus, according to the previous tests. Every seven days, ten vials were removed for PBS determination.

2.4 Statistical analysis

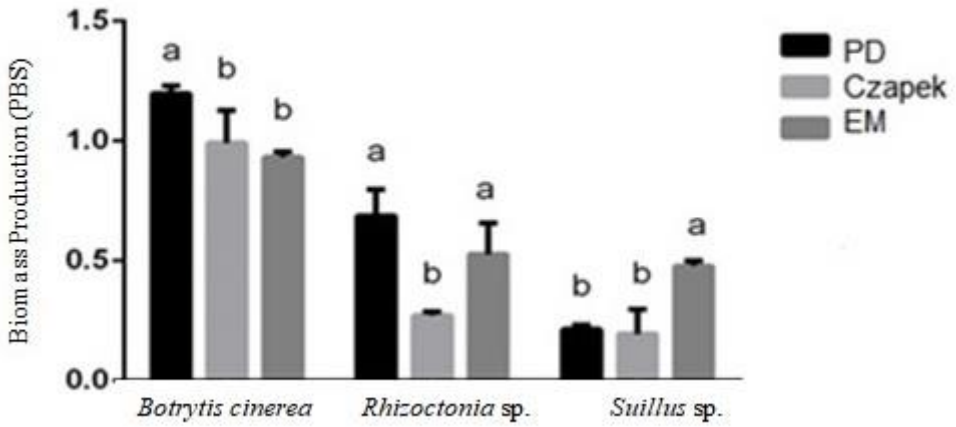
Statistical analysis was performed using IBM SPSS Statistics software version 20.0. The results were compared using the unidirectional and multivariate analysis of variance - ANOVA followed by Duncan's test for comparisons of variations. Mean, standard deviation (SD) and standard error were calculated for each of the samples. To determine the levels of statistical significance, a value of $p \leq 0.05$ was used.

3. Results and Discussion

3.1 Culture medium test

The PD medium determined the highest PBS for *B. cinerea* and *Rhizoctonia* sp. (Figure 1) and for *Suillus* sp. the largest PBS was in the EM medium.

Figure 1. Dry biomass (g / mL flasks) of *Botrytis cinerea*, *Rhizoctonia* sp. and *Suillus* sp. grown in Broth Potato Dextrose (PD), Broth Czapek and Broth Malt Extract (EM), for 35 days at 12, 16 and 20 ºC, respectively.



Source: The authors (2020).

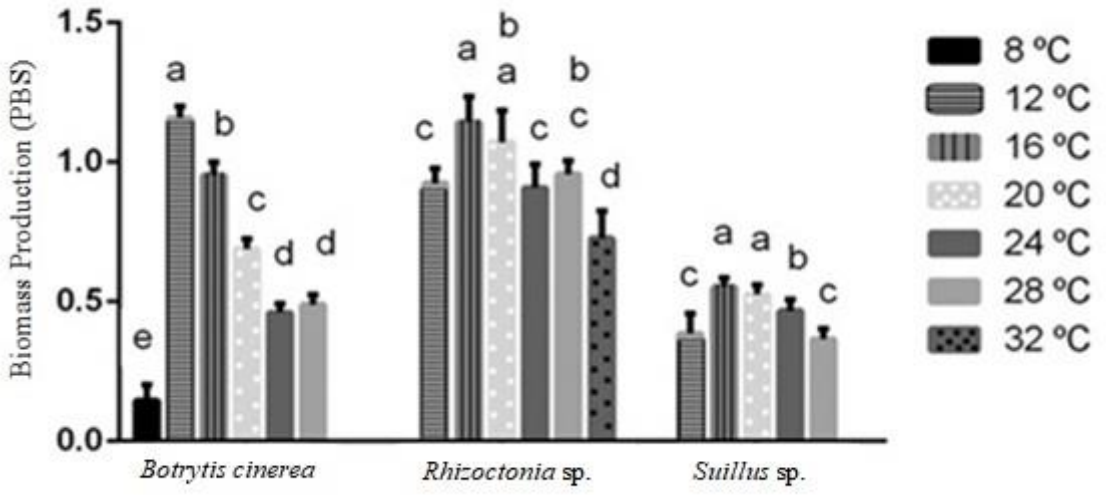
The CZ medium showed lower PBS for the three fungi, due to the fact that it is a culture medium that is low in carbon, vitamins and amino acids compared to potato and dextrose-based media (Peres, et al., 2003), thus implying in reduction of fungal biomass. The culture medium is the substrate on which the fungus develops and from the substrate consumed it can produce mycelium, CO_2, and several main metabolites such as alcohol, organic acids, extracellular polysaccharides and secondary metabolites (Papagianni, et al., 2004).

The culture media favor the growth of fungi according to their composition. Natural means based on potato and dextrose (PD broth) stand out for providing a good source of carbohydrates, favoring the growth and sporulation of fungi (Queiroz, et al., 2004). The applicability of this medium in mycelium cultures with pathogens of the genera *Botrytis* and *Rhizoctonia* was observed (Wu, et al., 2019; Jang, et al., 2018).

3.2 Cultivation temperature test

For *B. cinerea*, it was confirmed that the highest PBS occurred at a temperature of 12º C, observing the total absence of growth of the fungus at 32 ºC (Figure 2). In the case of *Rhizoctonia* sp. and *Suillus* sp. a higher PBS was observed at temperatures of 16 and 20 ºC, respectively (Figure 2). Like *B. cinerea*, *Suillus* sp. neither did it develop at 32 ºC (Figure 2).

Figure 2. Dry biomass (g / mL flasks) produced by *Botrytis cinerea* and *Rhizoctonia* sp. in Broth Potato-Dextrose (PD) and *Suillus* sp. in Malt Extract (EM) broth, under different temperatures (8, 12, 16, 20, 24, 28, and 32 ºC) for 35 days.



Source: The authors (2020).

Only for this fungus was the temperature of 8 ºC tested, as it grew well at 12 ° C, thus establishing the need to assess whether this fungus would be able to produce a greater amount of mycelium at lower temperatures. Studies by Lahlali et al. (2007) revealed that the radial growth of *B. cinerea* mycelium in PDA solid medium occurred between 5 and 25 ºC and that other factors such as water activity in the medium may be more important than temperature in the production of mycelium.

Sánchez et al. (2001) reported that *Suillus granulatus* (L.: Fr.) Roussel, *Suillus luteus* (L.: Fr.) Roussel and *Suillus collinitus* (Fr.) Kuntze had maximum dry biomass production at 23 ºC higher than the present study and only *S. collinitus* developed at 30 ºC. For *R. solani*, Ritchie et al. (2009) reported that radial growth of the mycelium in PDA solid medium occurred between 5 and 30 ºC, with optimal temperatures between 20 and 25 ° C, depending on the isolate.

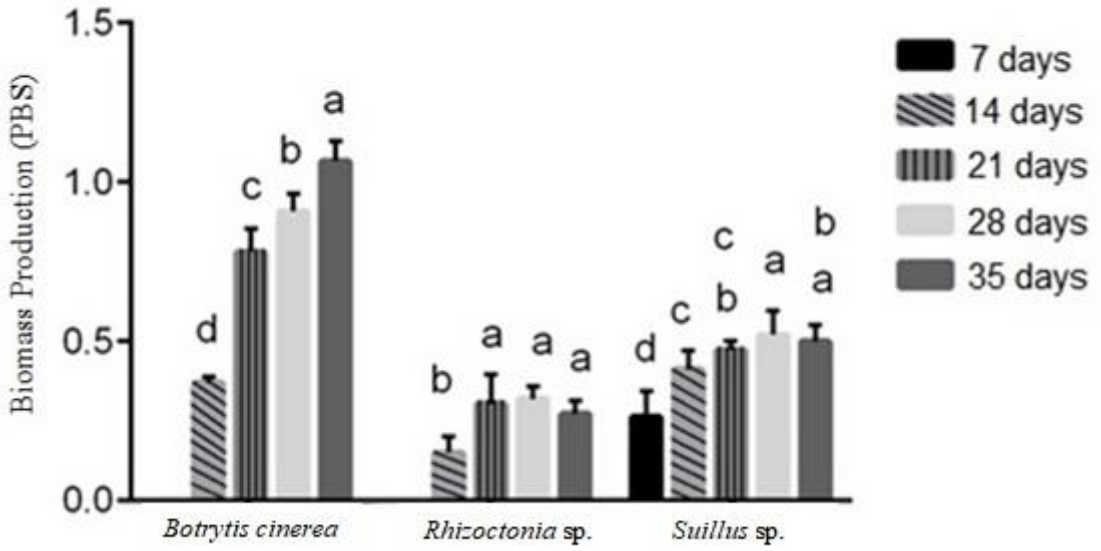
Fungi showed better development at lower temperatures (12, 16 and 20 ºC; *B. cinerea*, *Rhizoctonia* sp. and *Suillus* sp., respectively), with a gradual reduction beyond the optimal growth temperature. This condition of development at lower temperatures could reflect an ecological adaptation to the site average temperature where they were from. According to Climate-Data Org. (2020) average temperatures for Guarapuava/PR, Itapeva/SP, Colombo/PR are 16.7 ºC, 18.2 ºC and 16.6 ºC, respectively. Knowing the optimal growth temperature is

important for standardizing the production of mycelial mass of fungi, on a large scale (Rossi, et al., 2007). Temperature can also affect other important factors in liquid culture, such as dissolved oxygen and pH (Papagianni, 2004).

3.3 Cultivation time test

For *B. cinerea*, there was no growth in the first 7 days and continuous growth up to 35 days, when the highest PBS occurred (Figure 3). *Rhizoctonia* sp. neither did it grow in 7 days and the highest PBS occurred at 28 days. *Suillus* sp. grew at 7 days and the maximum PBS was also at 28 days. *Rhizoctonia* sp. and *Suillus* sp. had a decline in mycelial mass production after 28 days of incubation, indicating that they started their phase of decline or death.

Figure 3. Dry biomass (g / mL flasks) of *Botrytis cinerea*, *Rhizoctonia* sp. and *Suillus* sp. grown in Potato Dextrose Broth (PD) and Malt Extract Broth (EM), for 7, 14, 21, 28 and 35 days.



Source: The authors (2020).

The maximum PBS content varied according to the species of fungus. Many substances such as secondary metabolites are produced at different stages of development to ensure the survival of the fungus, and various components of the medium are used in the exponential growth phase due to the great metabolic activity that occurs (Montini, et al., 2006). The stages of fungal growth are affected by levels of carbon and nitrogen, as well as

by the availability of oxygen and components of the medium (Pinto, et al., 2002; Papagianni, et al., 2004; Rossi, et al., 2007). These studies, above all, emphasize the importance of evaluating the best culture conditions, differences in fungal metabolism or ways of improving the search for metabolites with potential biological activity.

4. Conclusions

In the case of the studied fungi, the following *in vitro* culture conditions were obtained: PD medium, 12 ºC for 35 days for *B. cinerea*; PD medium or EM medium, 16 ºC for 28 days for *Rhizoctonia* sp.; and EM medium, 16 to 20 °C for 28 days for *Suillus* sp. These parameters can be used for stationary biomass production of these fungi to extract metabolites for use in several industrial sectors, in addition to contributing to the standardization of fungal growth conditions *in vitro*.

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