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Article/Artigo

Different culture media containing methyldopa for melanin production by *Cryptococcus* species

Avaliação de diferentes meios de cultura contendo metildopa para a produção de melanina por espécies de *Cryptococcus*

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ABSTRACT

Introduction: Melanin production by species of *Cryptococcus* is widely used to characterize *C. neoformans* complex in mycology laboratories. This study aims to test the efficacy of methyldopa from pharmaceutical tablet as a substrate for melanin production, to compare the production of melanin using different agar base added with methyldopa, and to compare the melanin produced in those media with that produced in Niger seed agar and sunflower seed agar by *C. neoformans*, *C. laurentii,* and *C. albidus*. Two isolates of each species, *C. neoformans, C. laurentii,* and *C. albidus*, and one of *Candida albicans* were used to experimentally detect conditions for melanin production. **Methods:** The following media were tested: Mueller-Hinton agar (MHA), brain and heart infusion agar (BHIA), blood agar base (BAB), and minimal medium agar (MMA), all added with methyldopa, and the media Niger seed agar (NSA) and sunflower seed agar (SSA). **Results:** All isolates grew in most of the culture media after 24h. Strains planted on media BAB and BHIA showed growth only after 48h. All isolates produced melanin in MMA, MHA, SSA, and NSA media. **Conclusions:** Methyldopa in the form pharmaceutical tablet can be used as a substrate for melanin production by *Cryptococcus* species; minimal medium plus methyldopa was more efficient than the BAB, MHA, and BHIA in the melanin production; and NSA and SSA, followed by MMA added with methyldopa, were more efficient than other media studied for melanin production by all strains studied.

Keywords: *Cryptococcus* sp. Melanin. Culture media. Methyldopa.

RESUMO

Introdução: A produção de melanina por espécies de *Cryptococcus* é uma característica amplamente utilizada em laboratórios de micologia para caracterização do complexo *C. neoformans*. O objetivo deste estudo foi verificar a eficácia da metildopa na forma farmacêutica de comprimido, como substrato para a produção de melanina por *Cryptococcus*, comparar diferentes bases de meios de cultura acrescidas de metildopa para produção de melanina e comparar o pigmento produzido nestes meios com o produzido em ágar Níger e ágar girassol por *C. neoformans*, *C. laurentii* e *C. albidus*. **Métodos:** Foram testados dois isolados de cada uma das espécies, *C. neoformans*, *C.laurentii* e *C.albidus*, e um de *C. albicans* para avaliar a produção de melanina nos meios de cultura ágar Müeller-Hinton (MH), ágar *brain heart infusion* (BHI), ágar base sangue (BS), meio mínimo (MM), todos acrescidos de metildopa, e ainda ágar girassol e ágar Níger. **Resultados:** Todos os isolados cresceram na maioria dos meios após 24h. O crescimento nos meios BS e BHI somente ocorreu após 48h. Todos os isolados produziram melanina nos meios MM, MH, girassol e Niger. **Conclusões:** A metildopa de origem farmacêutica pode ser utilizada como substrato para a produção de melanina por espécies de *Cryptococcus*; o MM acrescido de metildopa mostrou-se mais eficiente na produção de melanina do que os meios BS, MH e BHI; ágar girassol e ágar Níger seguidos de MM acrescido de metildopa foram os mais eficientes na produção de melanina pelos isolados estudados.

Palavras-chaves: *Cryptococcus* sp. Melanina. Meios de cultura. Metildopa.

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INTRODUCTION

Cryptococcus neoformans and *C. gattii* are the main species of the *Cryptococcus* genera involved in clinical cases of cryptococcosis. Other species are less common, but two of these, *C. albidus* and *C. laurentii*, represent about 80% of clinical isolates excluding *neoformans* and *gattii* species[1].

Infections caused by *C. neoformans* occur more frequently in immunocompromized individuals, while *C. gattii* is often found infecting immunocompetent ones[2]. Other species of the genus have been isolated mostly in individuals with some type of immunodeficiency[1].

The laboratory identification of medical important *Cryptococcus* species takes into account the particular characteristics of this genus. The majority are yeasts that produce capsules, are able to grow at 37°C, and produce enzymes urease and laccase. When cultured in media containing phenolic or polyphenolic substrates, they form a pigment called melanin[3]. Enzyme laccase present in yeast acts on these phenolic substrates generating quinones, which undergo a process of autopolimerization and turn into melanin. The dark pigment retained in the cell wall of the fungus is responsible for the color shown by the colonies[4,5]. Other species of *Cryptococcus* may also produce melanin in the media, but not so intensely as *C. neoformans* and *C. gattii*[6,7].

Colonies of melanin-producing *Cryptococcus* species show a display of colors varying from brown to black when grown in agar media such as sunflower seed agar (*Helianthus annus*), Niger seed agar (*Phalaris canariensis*), birdseed agar (*Guizotia abyssinica*), potato-carrot agar, and other chemically defined media as L-dopa and caffeic acid agar[7]. Some recent studies have shown the production of pigment in mustard seed and chilli pepper agar, *Pinus halepensis* seed and blackberry agar, and in media containing substrates methyldopa, epinephrine, and norepinephrine[8-12].

While the proposed natural resources are easily found and inexpensive, they undergo a complex preparation. So we proposed this study with the

following objectives: I) to test the efficacy of methyldopa from a pharmaceutical tablet as a substrate for melanin production; II) to compare the production of melanin using different agar base added with methyldopa; and III) to compare the melanin produced in those media with that produced in Niger seed agar and sunflower seed agar by *C. neoformans*, *C. laurentii,* and *C. albidus*.

METHODS

Culture media

The following media were experimentally tested: Mueller-Hinton agar (MHA) (Isofar; Duque de Caxias, State of Rio de Janeiro, Brazil), brain and heart infusion agar (BHIA) (Biobrás, Belo Horizonte, State of Minas Gerais, Brazil), blood agar base (BAB) (Biobrás, Belo Horizonte, MG, Brazil), Niger seed agar (NSA), and sunflower seed agar (SSA). The minimal medium (MMA) composition per liter was: 20g of bacteriological agar (BIOBRÁS; Belo Horizonte, MG, Brazil), 5g of ammonium sulfate (Vetec; Duque de Caxias, RJ, Brazil), 2g of glucose (Isofar; Duque de Caxias, RJ, Brazil), 1g of yeast extract (Vetec; Duque de Caxias, RJ, Brazil), and 0.2g/L of methyldopa. Methyldopa (MD) (Medley; Campinas, State of São Paulo, Brazil) was acquired in the form of pharmaceutical tablets, weighed, and pulverized with the aid of mortar and pestle. The MMA was prepared by weighing the reagents described plus methyldopa, the pH adjusted between 5 and 6, and then sterilized and distributed in plates. MHA (composition: meat infusion — 5g/L; hydrolyzed casein — 17.5g/L; cornflour — 1.5g/L; agar-agar — 15g/L), BHIA (composition: gelatin peptone — 10.5g/L; infuse of brain and heart — 6g/L; meat peptone — 11g/L; dextrose — 20g/L; sodium chloride — 5.0g/L; sodium phosphate biphasic — 2.5g/L), and BAB (composition: casein peptone — 17.5g/L; yeast extract — 4.12g/L; cornflour — 1.5g/L; bacteriological agar — 15g/L; sodium chloride — 5.0g/L) were prepared according to manufacturer's instructions. After the addition of methyldopa, the pH was adjusted between 5 and 6, and the complete medium was sterilized by autoclaving at 121ºC for 15 min and distributed in polystyrene, 90x15mm Petri dishes. The NSA and SSA were prepared by boiling 70 g of the respective seeds in 500 mL of distilled water for 30 min followed by processing in a blender and filtration through gauze. Twenty grams of bacteriological agar (BIOBRÁS, Belo Horizonte, MG, Brazil) were added to the filtrate, the volume adjusted to 1,000mL, sterilized by autoclaving at 121ºC for 15 min, and distributed in Petri dishes.

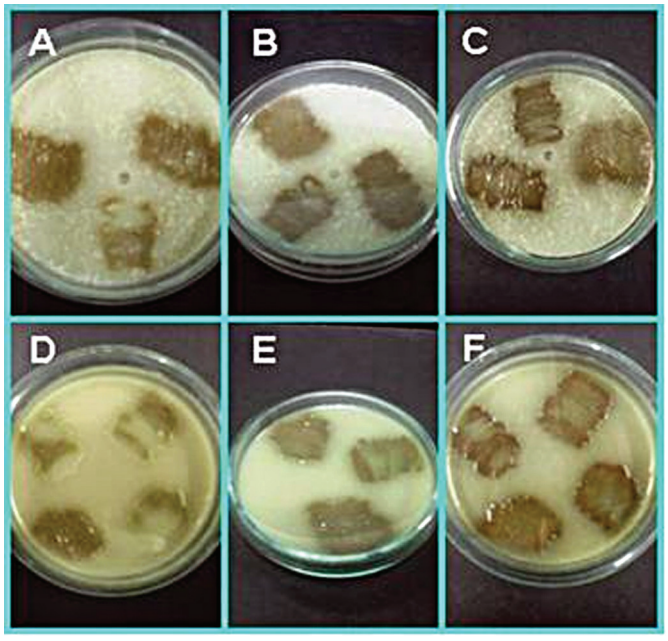

FIGURE 1 - Melanin produced by *Cryptococcus neoformans* in a traditional media for producing pigment.
A (24h), B (48h), and C (8 days): Sunflower seed agar. D (24h), E (48h), and F (8 days): Niger seed agar.

Microorganisms

The microorganisms used in the tests were two strains of *C. neoformans* (ATCC 90112, var. *grubii* and ATCC 28957, var. *neoformans*), two strains of *C. laurentii* (CRL05 and CRL12), two strains of *C. albidus* (CRA01 and CRA04), and one strain of *Candida albicans* (ATCC 64548), as a negative control for melanin production (previously tested and non-melanin producer). The microorganisms were kept at 4ºC in tubes containing Sabouraud dextrose agar (SDA), and subcultured every month.

According to the experimental design, portions of each microorganism colony (previously incubated at 30°C for 72h on SDA) were collected with a sterile disposable loop 1µL, and planted on the surface of the media under study. Four or five colony samples were planted on each plate. The tests were performed in duplicate and in two independent experiments. The plates were incubated at 30°C and observed at 24, 48, 72, and 96h and on the 8th day to check the variation of colony pigmentation.

The expression of results was performed by grouping the isolates with the same intensity of pigmentation and classifying them by the score: 0 (zero) for colonies with absence of pigments, 1 for a light brown, 2 for brown, and 3 for black colonies.

RESULTS

All isolates grew on most of the culture media studied after 24h of incubation. Isolates of *C. neoformans*, *C laurentii,* and *C. albidus* showed growth in BAB and BHIA only after 48h of incubation. **Table 1** shows the scores for melanin production by different isolates in the different media, according to incubation time.

The color of the colonies was stable from 4 to 8 days of incubation, for all isolates and on all media studied. Colonies of *C. albicans* showed no melanoid pigmentation, remaining colorless during all periods of incubation in all media. **Figures 1** and **2** show the melanin production in different media by the *Cryptococcus* strains and *C. albicans*.

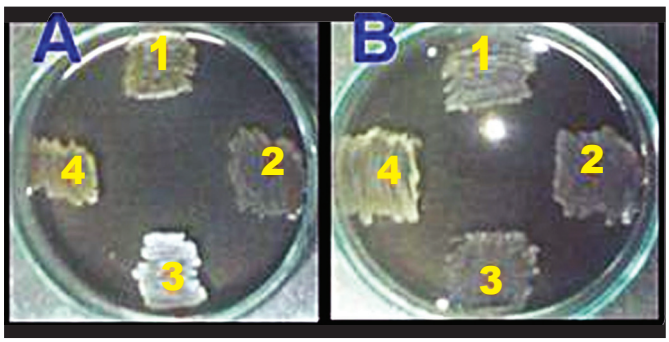

FIGURE 2 - Melanin produced by *Cryptococcus* spp. in a minimal medium containing methyldopa after 72h of incubation.
A) 1 and 4: *Cryptococcus neoformans*; 2: *Cryptococcus albidus*; 3: *Candida albicans;* B) 1 and 2: *Cryptococcus albidus*; 3: *Cryptococcus laurentii*; 4: *Cryptococcus neoformans.*

TABLE 1 - Production of melanin by *Cryptococcus neoformans*, *Cryptococcus laurentii,* and *Cryptococcus albidus* isolates, expressed as scores, in different culture media containing methyldopa after different times of incubation.

Isolates	Time																					
	24h					48h					72h					96h					
	BHIA	BAB	MMA	MHA	SSA	NSA	BHIA	BAB	MMA	MHA	SSA	NSA	BHIA	BAB	MMA	MHA	SSA	NSA	BHIA	BAB	MMA	MHA	SSA	NSA
90112	0	0	0	1	1	1	1	1	1	1	2	2	1	1	2	1	2	2	2	2	2	2	3	3
28957	0	0	0	1	1	1	1	1	2	1	2	2	1	1	2	1	2	2	2	2	2	2	3	3
CRA01	0	0	0	1	1	1	0	0	1	1	1	1	0	0	2	1	1	1	0	0	2	2	2	2
CRA04	0	0	1	1	1	1	0	0	1	1	1	1	1	1	2	1	1	1	1	1	2	2	2	2
CRL05	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	2	2	2	2
CRL12	0	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2
C. albicans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

BHIA: brain heart infusion agar, **BAB:** blood agar base, **MMA:** minimal medium agar, **MHA:** Mueller-Hinton agar, **SSA:** sunflower seed agar, **NSA:** niger seed agar, Scores: 0=no pigment, 1=light brown, 2=brown, and 3=black.

DISCUSSION

The concept of formulating differential and efficient culture media for the isolation and identification of pathogenic fungi, such as the genus *Cryptococcus*, is not new. Specific, differential, and selective media are often sought for certain groups, genera, or species of microorganisms. Thus, an ideal medium would facilitate the work of the mycologist, reducing the processing time of culture and identification of the fungus, besides the low cost and easy-to-prepare, -to-standardize, and -to-interpret results. However, many problems, such as a dubious and time-consuming interpretation of results, have hampered the achievement of this goal. In this way, many studies have been performed to formulate a culture medium that is effective for the presumptive identification and differentiation of isolates of the *C. neoformans* complex.

This study analyzed a culture medium composed by a few components, which were of low cost, easy to prepare, and able to show production of melanin by the species *C. neoformans*, *C. albidus,* and *C. laurentii*. The medium was prepared using methyldopa of pharmaceutical origin as a substrate for the melanin production; methyldopa was used because of its low cost and being available at the pharmacies in Brazil.

In laboratory practice, chemically defined media have been employed, with the advantage of known constituents, but media prepared with natural resources, such as Niger seed agar, are widely used by some authors[13]. In the present study, low concentrations of glucose and yeast extract were added to the culture media to provide the elements necessary to accelerate growth and melanin expression. However, this procedure was positive only for growth, as melanin production was not affected. The early oxidation of agar was inhibited and extended from as early as 24h to after 72h of incubation by the adjustment of pH between 5 and 6 that allowed better interpretation of the results.

Niger seed agar, sunflower seed agar and minimal medium agar showed growth and pigmentation of the isolates under study in a shorter period of time (24-72h). BHIA, BAB, and MHA media plus methyldopa showed slight change in color of the colonies of *C. neoformans* after 48 h of incubation. The *C. albicans* strain did not produce dark-pigmented melanin during the study. This is important to be mentioned because *C. albicans* is able to produce melanin under certain laboratory conditions[14]. Similar results to those presented with SSA, NSA, and MMA were observed by Mseddi et al.[12] in both the *Pinus halepensis* agar and the blackberry agar, and by Pedroso et al.[7] in the L-dopa agar. Hernández et al.[9] also evaluated the production of melanin in sunflower seed agar by *C. neoformans* isolates and found that most isolates produced an intense pigmentation.

The culture media used in this study appear to give faster results in the expression of melanin than the *Mucuna pruriens* agar[15]. In this medium, the authors showed that colonies of *C. neoformans* changed colors only between the third and fifth days of incubation. In another recent study[12] using seeds of *Pinus halepensis* and blackberry in the production of a new culture media for isolation of *Cryptococcus* species, the results obtained were satisfactory, and the medium was simple to prepare and enabled a rapid and presumptive identification of *C. neoformans*.

Comparison of melanin production among isolates of *Cryptococcus* species revealed that *C. neoformans* isolates formed colonies with stronger color in a shorter time than the isolates of *C. laurentii* and *C. albidus*, in all media. According to Ikeda et al.,[6] this occurs because the enzyme activity of phenoloxidase in all strains of the genus *Cryptococcus* other than the *C. neoformans* complex is lower, including in several clinically emerging species that express melanin. Regarding the identification of *C. neoformans*, the melanin pigment in Niger seed agar, sunflower seed agar, and minimal medium agar supplemented with methyldopa, was a good indicator of presumptive identification in 24-72h of incubation.

We concluded that: I) methyldopa in the form pharmaceutical tablet can be used as a substrate for melanin production by *Cryptococcus* species; II) minimal medium plus methyldopa was more efficient than the BAB, MHA, and BHIA in the melanin production by strains studied; and III) NSA and SSA, followed by MMA, added with methyldopa were more efficient than the other media studied in providing evidence of melanin production by *C. neoformans*, *C. laurentii,* and *C. albidus* strains studied.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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