

Toxigenic potential of *Aspergillus flavus* tested in different culture conditions

Potencial toxigênico de *Aspergillus flavus* testado em diferentes meios e condições

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Abstract

The objective of the present work was to evaluate the capacity of three isolates of *Aspergillus flavus* to produce aflatoxin under different culture conditions. This experiment was based on a 2³ factorial design, in which the independent variables were temperature (20-40 °C), incubation time (7-21 days), and the pH (2.0-6.0) in two different synthetic media. The optimal conditions were applied to non-aflatoxigenic isolates previously tested in coconut agar. Aflatoxin B₁ was extracted directly from the synthetic cultures with chloroform. Thin Layer Chromatography (TLC) and Photographic Photometry were utilized to identify and quantify the compounds. Preliminary results showed that YES agar was an alternative medium for detecting the toxigenic potential of *Aspergillus flavus* in the following conditions: pH of 5.2, temperature of 25 °C, and incubation time of 11 days producing 206.05 ng.CFU⁻¹ of aflatoxin B₁. Of the 30 non-aflatoxigenic isolates, 12 presented a positive result in the optimal media and conditions tested.

Keywords: *Aspergillus flavus*; aflatoxin-producing; incubation time; temperature; pH; culture media.

Resumo

O objetivo do presente trabalho foi avaliar a capacidade produtora de aflatoxina de três isolados de *Aspergillus flavus* sob diferentes condições de crescimento. O experimento foi baseado num delineamento experimental 2³, tendo como variáveis independentes a temperatura (20-40 °C), tempo de incubação (7-21 dias) e pH (2,0 e 6,0) em dois meios de cultura diferentes. As melhores condições encontradas foram empregadas com isolados não toxigênicos testados previamente em Agar Coco. A aflatoxina B₁ foi extraída com clorofórmio, diretamente dos meios sintéticos. A identificação e a quantificação do composto foram efetuadas por Cromatografia em Camada Delgada e Fotometria Fotográfica. Como resultados preliminares, o meio de cultura YES se mostrou como uma alternativa para detectar o potencial toxigênico de *Aspergillus flavus*, nas seguintes condições: pH 5,2, temperatura de 25 °C e tempo de incubação de 11 dias, com uma produção de 206,05 ng.UFC⁻¹ de aflatoxina B₁. Dos 30 isolados não toxigênicos, 12 apresentaram resultado positivo nas condições e meios de cultura testados.

Palavras-chave: *Aspergillus flavus*; aflatoxina; tempo de incubação; temperatura; pH; meios de cultura.

1 Introduction

Mycotoxins are secondary metabolites that are produced by certain filamentous fungi (SWEENEY; DOBSON, 1998; D'MELLO; MACDONALD, 2002; GIRAY et al., 2007). More than 20 species of *Aspergillus* produce mycotoxins; however, the most easily found are those from the *flavi* section, which includes three species: *A. flavus*, *A. parasiticus* and *A. nomius* (FRISVAD et al., 2006; IQBAL et al., 2006).

The four aflatoxins that are produced naturally are B₁, B₂, G₁, and G₂; B₁ is the most commonly found in high concentrations in food and feed (SWEENEY; DOBSON, 1998; VAAMONDE et al., 2003). However, not all strains of *A. flavus* produce aflatoxins; since several strains are non-toxigenic aflatoxin, tsynthesis may become unstable in this species (ORDAZ et al., 2003).

Aflatoxins are compounds with a hepatotoxic, carcinogenic, and teratogenic potential that can affect either humans or animals (MOSS, 2002; SALEEMULLAH et al., 2006; D'MELLO; MACDONALD, 2002). According to an IARC estimates (INTERNATIONAL..., 2002), aflatoxins can be considered carcinogenic to humans (group I).

The conditions that promote mycotoxin production are usually more restricted than those for mould growth (GUITAKOU et al., 2006; BRESLER et al., 1995). Mycotoxin production depends on fungal species, substrate, temperature of the media, pH, relative humidity, and storage or incubation time (BRAGULAT et al., 2001; FILTENBORG et al., 1983; SMEDSGAARD, 1997; BELLI et al., 2004; WEELER et al., 1991).

According to Park and Bullermann (1983), substrate is one of the most important factors controlling the production of secondary metabolites. In addition, the optimal medium for production of secondary metabolites by fungi depends on the metabolite, species, and isolate (BRAGULAT et al., 2001; MEDINA et al., 2006). For example, different strains of *A. flavus* have been shown to produce aflatoxins at different rates when cultured under similar conditions (HUSSEIN; BRASEL, 2001; FILTENBORG et al., 1996).

Some specially developed media can identify toxigenic strains of *Aspergillus*, such as *Aspergillus flavus* and *parasiticus* agar (AFPA) (PITT et al., 1983). Another approach to the

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characterization of strains of *A. flavus* and *A. parasiticus*, both aflatoxin producers, was developed by Lin and Dianese (1976). Although it is a simple and useful technique, some false negative results may be detected (TANIWAKI, 1996).

Considering the importance of evaluating alternative and efficient methods for the characterization of toxigenic potential, the objectives of this study were to evaluate the growth capacity and toxigenic potential of three *Aspergillus flavus* strains isolated from rice (*Oryza sativa* L.) in different culture conditions.

2 Materials and methods

2.1 Fungal isolation and identification

All fungi tested in this study were isolated from rice (*Oryza sativa* L.) and identified according to Klich and Pitt (1988) by Hoeltz (2005) during the years 2003 and 2004. A total of 33 strains of *A. flavus* were used in the experiment. According to the method developed by Lin and Dianese (1976), three proved to be aflatoxigenic. Those three isolates were classified as *A. flavus* A43, A46, and A21.

2.2 Culture media

The following culture media were used in the present work: YES: 20 g yeast extract, 150 g saccharose, 20 g agar, 0.5 g magnesium sulphate, and 100 mL distilled water; CYA: 5.0 g yeast extract, 30.0 g saccharose, 15.0 g agar, 10.0 mL concentrated Czapeck, 1.0 g H₂HPO₄, and 100 mL distilled water; coconut agar: 600 mL coconut milk, 200 mL distilled water, and 16 g agar. The pH of the media was adjusted to 2.0, 2.8, 4.0, 5.2, and 6.0 using 10% tartaric acid. The media were autoclaved at 121 °C for 15 minutes and poured into 90 mm Petri dishes, which held approximately 15 mL of media.

2.3 Culture conditions

Fungi were grown in Sabouraud agar (Merck) for 7 days at 25 °C to obtain heavy sporulation. Spore suspensions were obtained by harvesting spores of each isolate and suspending them in sterile distilled water containing 0.005% of a wetting agent (Tween 80). CYA and YES agar plates were needle-inoculated centrally with each spore suspension and finally incubated at the required temperature (20, 25, 30, 35, and 40 °C) for 7-21 days.

2.4 Qualitative analysis of aflatoxin B₁

After the appropriate incubation times, the mycelium of each fungal colony was scraped off the Petri dish, cut into strips, and homogenized with approximately 5 mL of chloroform (FITELBORG et al., 1983, with modifications). The extracts were applied to plates of silica gel 60G (Merck) using capillaries and were eluted with toluene, chloroform, ethyl acetate, and formic acid (35:25:25:10). After developing the chromatogram, the plates were viewed under long-wavelength ultraviolet light (365 nm) to detect the characteristic fluorescence of aflatoxin B₁ (LIN; DIANESE, 1976).

2.5 Quantification of aflatoxin B₁

The samples to be quantified were applied to the plates with 25, 20, 15, and 10 ng pattern of aflatoxin B₁ and 80, 60, 40 and 20 µL of each sample. The chromatographic plates were eluted with chloroform-acetone (90:10) (BRASIL, 2005). The fluorescent spots visualized under UV light were analysed using an Olympus photometric camera with a 5.1 megapixel CCD detector. Ten photographs of each sample were taken; five with the left standard and five with the right standard. Spots and fluorescence intensity were measured using the software IMSTAT (image statistics) from the IRAF (Image Reduction Astronomical Facility) package following the standard procedures used in Astronomy to measure source brightness (LANCASTER et al., 2005). IMSTAT allows the plate background to be discounted by measuring the surrounding area of each spot to obtain the intrinsic brightness of the spots. In all cases, the standard curve was fitted by linear regression with a correlation coefficient higher than 0.95. The ten values of each sample fluorescent AFB₁ spot intensity were promediated and interpolated in the standard curve.

The detection limit was tested by the application of 2, 4, 6 and 8 µL of aflatoxin B₁ standard solution corresponding to 1, 2, 3 and 4 ng aflatoxin.

2.6 Determination of the aflatoxin-producing ability of non-aflatoxigenic isolates

Thirty non-aflatoxigenic isolates previously tested in coconut agar (LIN; DIANESE, 1976) were tested using the optimal conditions determined above, on both media (CYA and YES). The identity of aflatoxin B₁ was confirmed using the method of Przybylski (1975), and the aflatoxigenic potential was tested again on coconut agar medium.

2.7 Statistical analysis

In order to carry out this experiment, a central composite planning constituted of a factorial 2³ with three central points and six axial points resulting in 17 experiments (RODRIGUES, 2005). The central composite design was used to study the effect of temperature, pH, incubation time, and their interaction. The culture media were analyzed by response surface methodology using Statistica 7.0 (Statsoft, USA) software resulting in three Pareto graphics.

3 Results and discussion

3.1 Interaction of independent variables (pH, temperature, and incubation time)

The three isolates A43, A46, and A21 tested in this work were aflatoxigenic in different conditions, as shown in Table 1. In some cases, two isolates presented the same behaviour and produced aflatoxin B₁ in the same conditions of pH, time, temperature, and culture media (CYA and YES).

The isolates A43 and A21 produced aflatoxin B₁ in the two following conditions: at 20 °C with a pH of 4.0 and incubation time of 14 days, and at 25 °C with a pH of 5.2 and an incubation

Table 1. Independent Variables Interaction (pH, temperature, and incubation time) and quantification analyses.

	°C	pH	Time (days)	A43	YES A46	A21	A43	CYA A46	A21
				Quant. (ng)	Quant. (ng)	Quant. (ng)	Quant. (ng)	Quant. (ng)	Quant. (ng)
1	25	2,8	11	-	-	-	-	-	-
2	35	2,8	11	-	-	-	-	-	-
3	25	5,2	11	206,05	-	-	35,2	-	-
4	35	5,2	11	-	-	-	-	-	-
5	25	2,8	18	-	-	-	-	-	-
6	35	2,8	18	-	-	-	-	-	-
7	25	5,2	18	56,65	-	40,0	68,85	-	178,0
8	35	5,2	18	-	-	-	-	-	-
9	30	4	14	-	-	-	-	-	-
10	30	4	14	-	-	-	-	-	-
11	30	4	14	-	-	-	-	-	-
12	20	4	14	18,85	17,0	40,1	42,4	-	52,58
13	40	4	14	-	-	-	-	-	-
14	30	2	14	-	-	-	-	-	-
15	30	6	14	-	-	-	-	-	-
16	30	4	7	-	-	-	-	-	-
17	30	4	21	-	-	-	-	-	-

time of 18 days. Only one isolate, A46, did not show any signs of toxicity in the combinations of the conditions analysed when grown on CYA medium. This isolate also produced aflatoxin B₁ in the smallest number of combinations.

With regard to the different temperature values tested (20, 25, 30, 35 and 40 °C), only 20 and 25 °C proved to be appropriate for aflatoxin B₁ production.

These results conflict with those of Gqaleni et al. (1997), who reported that the optimal temperature for the production of aflatoxin was 30 °C using the CYA and YES media.

The production of aflatoxin B₁ occurred only at pH 4.0 and 5.2, and pH 5.2 appeared most frequently in the combinations in which the isolates were aflatoxin B₁-producers. Molina and Giamuzzi (2002) studied the effect of pH (5.5 and 5.9) on the production of aflatoxin B₁, and reported that pH 5.9 gave the highest aflatoxin levels.

The minimum period of incubation necessary for the production of aflatoxin B₁ was eleven days. However, fourteen and 18 days were the periods of time during which the production reached the highest number of combinations. Frisvad and Filtenborg (1983) analysed *Aspergillus* cultures in CYA and YES media for a period of 7, 9, 12, and 14 days of incubation, and concluded that 14 days was the optimal length of time for mycotoxin development.

Gqaleni et al. (1996) and Leontopoulos et al. (2003) reported that aflatoxin production was observed after the third day of incubation on YES medium. A year later, Gqaleni et al. (1997) reported that aflatoxins were observed after 5 days incubation of an *Aspergillus flavus* isolate on CYA and YES media, and that aflatoxin production reached the maximum rate at the end of 15 days incubation. Park and Bullerman (1983) inoculated spores of *A. flavus* and *A. parasiticus* in cheese and rice and analysed the production of aflatoxin B₁ over the

following periods: 7, 10, 14, 21, and 28 days. They concluded that aflatoxin production reached the maximum rate at 14 days.

3.2 The effects of culture media on aflatoxin B₁ production

Qualitative analysis

The three tested isolates differed when inoculated under the same conditions on different media. The A43 isolate, on CYA and YES, produced aflatoxin B₁ at 25 °C, pH 5.2, and 11 days incubation time. The A46 isolate produced aflatoxin B₁ only on YES medium (20 °C, pH 4.0, and 14 days). These data suggest an advantage of the YES medium when compared to the CYA medium.

Abranson and Clear (1996) tested both media for mycotoxin production and reported that CYA agar was slightly better than YES agar. This may be due to the amount of saccharose in the media (15% in YES and 3% in CYA); this can lead to a less hydrophilic and more permeable layer for lipophilic solvents allowing better extraction of the secondary metabolites from the CYA medium.

Bragulat et al. (2001) and Frisvad and Filtenborg (1983) reported that the CYA and YES media should be used together for the detection of toxigenic potential in fungi since they seemed to allow the production of different mycotoxins.

Quantitative analysis

To quantify the production of aflatoxin B₁ by the three tested isolates, the same conditions and experimental design were used but only with the combinations of conditions in which the isolates produced toxins. The experiments were replicated twice and the averages were analysed statistically (Table 1) using the software Statistica 7.0.

The isolate A43 inoculated in YES medium at pH 5.2, 25 °C and with an incubation time of 11 days produced the highest levels of aflatoxin B₁ (206.05 ng.CFU⁻¹). When inoculated in CYA medium, this same isolate produced much lower levels (35.2 ng.CFU⁻¹) under the same conditions Gqaleni et al. (1997) reported the production of 0.226 µg.mL⁻¹ in YES medium at an a_w of 0.95 and 0.183 µg.mL⁻¹ in CYA medium. It should be noted that the combinations of the conditions analysed in this study differed from those used by Gqaleni, who did not consider the pH in the media, but did consider their water activity.

On the YES medium, an incubation time of 11 days promoted the highest production of aflatoxin B₁ (206.05 ng.CFU⁻¹); on the CYA medium, the best results were produced after 18 days of incubation (178 ng.CFU⁻¹), when the A21 isolate was incubated at a pH of 5.2 and a temperature of 25 °C.

After 14 days of incubation on YES medium, the concentration of aflatoxin was slightly lower compared with the two other periods (11 and 18 days). These results differ from those found by Gqaleni et al. (1996), who concluded that the aflatoxin detected on the YES medium was produced from the second day of incubation and reached its highest concentration (130 µg.g⁻¹) after 15 days; the concentration subsequently decreased to 75 µg.g⁻¹ after 21 days.

The production of aflatoxin B₁ by isolate A21 on the YES medium was positively influenced by pH (p = 0.1). This means that as the pH increased the production of the mycotoxin increased (Figure 1).

When analysed individually, the pH effect was significantly negative and the temperature was significantly positive for the isolates A43 and A21, when inoculated on CYA (Figure 2 and 3). This means that if the pH is reduced and the incubation time is increased, the production of aflatoxin B₁ may be higher.

On the CYA medium, the production capacity of the same strain was influenced by the interaction of all factors tested when analysed together (p = 0.5) (Figure 3).

3.3 Aflatoxin-producing ability

Thirty isolates thought to be non-aflatoxin producers (HOELTZ, 2005) were tested on coconut agar in the optimal conditions determined in this study. Of these, 12 produced aflatoxin B₁ (Table 2).

We found that ten isolates (1, 3, 4, 5, 7, 9, 12, 8, 10 and 11) proved to be producers in the same conditions (20 °C, pH 4.0, and 14 days of incubation); three in CYA (8, 10 and 11) and four in YES media (5, 7, 9, and 12). Three isolates (1, 3 and 4) were producers at 20 °C, pH 4.0, and 14 days of incubation, on both media. Two isolates (2 and 6) were producers at 25 °C, pH 5.2, and 18 days of incubation, both on CYA and YES media. Isolate 2 also produced aflatoxin B₁ at 25 °C, pH 5.2, and 11 days of incubation on the YES medium.

Interestingly, the aflatoxin-producing isolates at pH 4.0 and 20 °C appeared to be non-producing isolates at pH 5.2 and 25 °C. This reveals an advantage of the YES medium because more isolates produced aflatoxin B₁ when inoculated on this

medium under a large range of conditions (25 °C, pH 5.2, 11 days; 25 °C, pH 5.2, 18 days; and 20 °C, pH 4.0, and 14 days). Leontopoulos et al. (2003) concluded that YES agar is a great

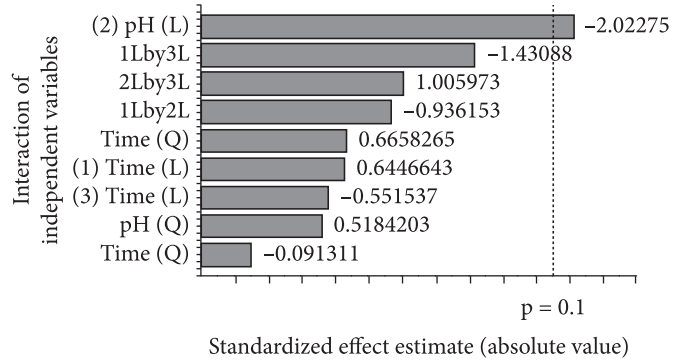


Figure 1. Pareto graphic showing the positive influence (p = 0.1) of the pH on aflatoxin B₁ production by isolate A21 in the YES medium, where 1 represents the temperature, 2 represents the pH, and 3 represents the incubation time.

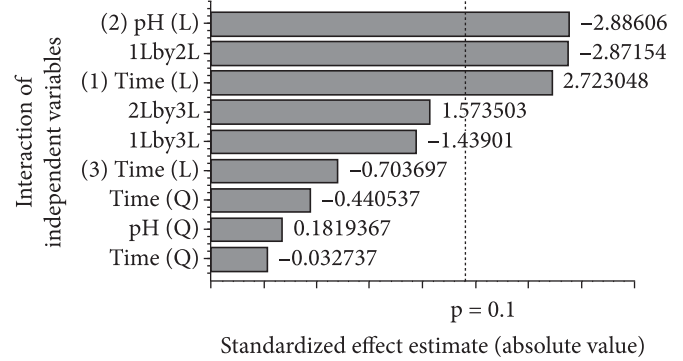


Figure 2. Pareto graphic showing the negative influence of pH and positive influence of temperature (p = 0.5) on aflatoxin B₁ production by isolate A43 in the CYA medium, where 1 represents the temperature, 2 represents the pH, and 3 represents the incubation time .

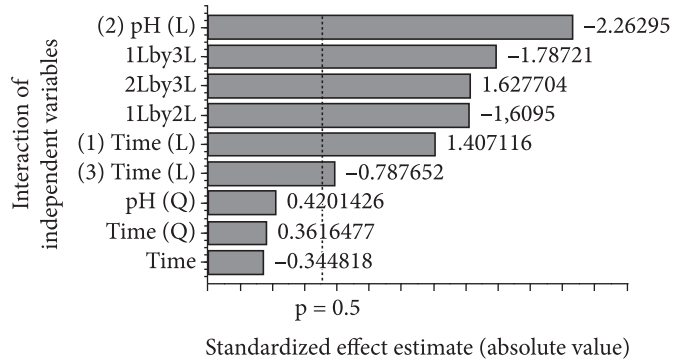


Figure 3. Pareto graphic showing the significant influence (p = 0.5) of the interaction between time, temperature, and pH on aflatoxin B₁ production by isolate A21 in the CYA medium, where 1 represents the temperature, 2 represents the pH, and 3 represents the incubation time.

Table 2. Aflatoxin-producing ability: Analysis of non-aflatoxigenic isolates under different conditions.

Condition tested	Medium	pH	°C	Time (days)	Isolates produced
A	YES	5,2	25	11	2
B	YES	5,2	25	18	2 e 6
C	YES	4,0	20	14	1, 3, 4, 5, 7, 9, 12
D	CYA	5,2	25	11	-
E	CYA	5,2	25	18	2 e 6
F	CYA	4,0	20	14	1, 3, 4, 8, 10, 11

medium for the biosynthesis of aflatoxin B₁, which proved easy to prepare and relatively cheap.

Ordaz et al. (2003) used new media (CYA and YES + 0.6% of YCSD) to detect aflatoxigenic potential and verified that all isolates, initially toxigenic, produced a fluorescent ring under UV light. Twenty-eight non-aflatoxigenic isolates were subsequently tested on these media and none presented the fluorescent ring. The analyses of aflatoxins by HPLC were also negative.

Different strains of *A. flavus* may produce aflatoxins in different proportions when cultivated under similar conditions (HUSSEIN; BRASEL, 2001). According to Vaamonde et al. (2003), data from different geographic areas show the largest potential variation in mycotoxin-producing *Aspergillus*.

The isolates that were initially thought to be non-aflatoxigenic but proved to be producers under the conditions and media tested in this study were tested again on coconut agar. Eight isolates still showed negative results on this medium. These data are similar to those reported by Taniwaki (1996) who described coconut agar (LIN; DIANESE, 1976) as unreliable for the detection of the toxigenic potential of *A. flavus* and *A. parasiticus* aflatoxin due to false negative results.

4 Conclusions

Isolate A43 produced aflatoxin B₁ in a large variety of conditions and also produced the highest concentration of aflatoxin B₁ determined by the quantitative tests (206.4 ng.CFU⁻¹). The thin layer method proved to be very efficient in detecting aflatoxin B₁ generating highly satisfactory results in the quantitative analysis utilizing TLC with Photometric Measurements. The detection of the toxigenic potential of isolates of *A. flavus* on coconut agar is not efficient since some toxin-producing strains were not identified. As an alternative, the results of the present study suggest the use of the YES medium at 25 °C, at a pH of 5.2, and with an incubation time of 11 days to detect the toxigenic potential of *A. flavus*.

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