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Studies on *Aspergillus flavus* growth and toxicity

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Abstract

Aflatoxins are secondary metabolites of Aspergillus flavus and Aspergillus parasiticus molds. They are highly toxic, mutagenic, teratogenic and carcinogenic compounds which contaminate a great variety of important agricultural products such as peanuts, maize, rice and cottonseeds. Aflatoxins are found in tropical and subtropical regions. Nowadays there is a global concern for the removal of these toxins from food. In order to assure a food security, the article reviews some aspects of molds growth and production of toxins.

Keywords: *A.flavus*, growth modelling, toxinogenesis mechanism

Introduction

Micotoxins are produced by five types of molds: *Aspergillus, Penicillium, Fusarium, Claviceps* and *Alternaria* [2,14]. *A. flavus, A. parasiticus,* [5,7], *A. nomius* and *A. tamarii* [4,10] are molds responsible for the production of different secondary metabolites from the family of aflatoxins. There are four major types of aflatoxins: B1, B2, G1, G2 as well as two other metabolic products excreted in milk: M1 and M2. Several studies showed that aflatoxin B1 is the most toxic and carcinogenic [13].

According to Mannon and Johnson (1985)[8] around 25% of food products are contaminated with mycotoxins. Every year almost 55 billion tones of cereals are lost in the world due of this contamination.

A very important goal in the agricultural and food field is to maintain the quality of the food products and to guaranty the consumers' health. Food industry involves a succession of technological transformations and the final product must present a minimum microbiological risk. To obtain this minimum risk it has been developed a system which controls the critical points HACCP (Hazard Analysis. Critical Control Points.) [15]. An alternative method used also for predictive microbiology evaluates the developing speed of microorganisms using mathematical methods. For a long time this method has been concentrated on the study of bacteria but there is a tendency to extend the method on molds [3]. It is difficult to measure in the same time the growth of fungi and the production of toxins on agar medium.

Molecular studies have been made on different species of *Aspergillus* on the aflatoxin synthesis pathways; on the other hand, the production of these secondary metabolites hasn't received the same attention concerning the modelling. The sterigmatocystin is synthesized through the same metabolic pathway as the aflatoxins [1,6]. The genes active in the metabolic pathway of aflatoxin synthesis are similarly organized in *A. flavus, A. parasiticus* and *A. nidulans* [16].

In *Emericella nidulans* (sexual form of *A. nidulans*) the gene *fluG* has been identified and sequenced. The product of this gene is the FluG protein involved in the initiation of the

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conidiation process, inducing the synthesis of an extracellular factor and inhibiting in the same time the growth of the mycelium. FluG C-terminal-domain is structurally and functionally similar to glutamine-syntase type I (GSI).

Two approaches are included in this study: the molecular analysis regarding the presence of an ortologue *fluG* gene from *A. nidulans* in *A. flavus* and, respectively, modelling of mycelium growth of three *A. flavus* strains grown under different temperatures.

1. Materials and methods

1.1. Modelling

Three strains of *A. flavus* have been used*:* two of them produce aflatoxins LABO 10038, MUCL 18903 and the third one does not produce any toxic compounds NRRL 6550.

All three have been inoculated on MEA medium (Malt Extract 20%, Glucose 20%, Peptone 1%, Agar 20%) and grown at 27°C in order to obtain spores for further inoculations. After 7 days, the spores have been preloaded in sterile physiological water (9 $g.L^{-1}$ de NaCl), counted, diluted if necessary and used as an inoculum. After the inoculation on MEA medium, the Petri dishes were put in hermetically closed recipients with water added to maintain the humidity.

Because mycelium growth depends on the temperature, for the modeling each recipient has been incubated at 13 temperatures values ranging from 5°C to 36,5°C (5 ; 6,5 ; 8,5 ; 10,5 ; 12,5 ; 14,5 ; 18 ; 22,5 ; 29 ; 31,5 ; 33 ; 35 ; 36,5). The mycelium growth has been daily monitored by analysing the colonies in each Petri dish. Measurement of the two perpendicular diameters of individual colonies allowed estimation of the average radius. Using *Slide Write® Plus Version 5* software, results of the mycelium growth (growth rate) were further transformed in a secondary model.

The ROSSO secondary model (Rosso & Robinson, 2001; Sautour & *colab*, 2001) aims to describe the temperature's influence (X=T), on *A. flavus* developing.

1.2. Molecular study

Experiments started with the isolation and purification of fungi DNA using the method described by Mayer *& colab.* (2003)[9]. In order to determine the presence of a *fluG* gene at *A. flavus*, special primers of this gene from *A. nidulans* (*GenBank accession number L27817*) have been designed starting from the coding sequence. PCR amplification reaction involved denaturation (94°C, 5 min), hybridasation (45°C, 30 sec) and elongation (72°C, 1 min). The control primers are S1 and S2.

S1 5'ATTGGAGGGCAAGTCTGGTG-3'
S2 5'CCGATCCCTAGTCGGCATAG-3

Next the fragments of DNA have been separated by electrophoreses.

2. Results and discussions

2.1. Modeling

The modeling results show that temperature influences the growth rate of all three *Aspergillus flavus* strains in the same manner. The optimal growth temperature for *Aspergillus flavus* is around 31°C, value determined by other studies (Rollet, 2004)[11].

The growth curve shape is asymmetrical and identical for the three strains and this curve is (Figure 1). In the range between the minimum temperature (Tmin) and the optimal temperature (Topt) the curve constantly increases, while from Topt it brutally drops. The

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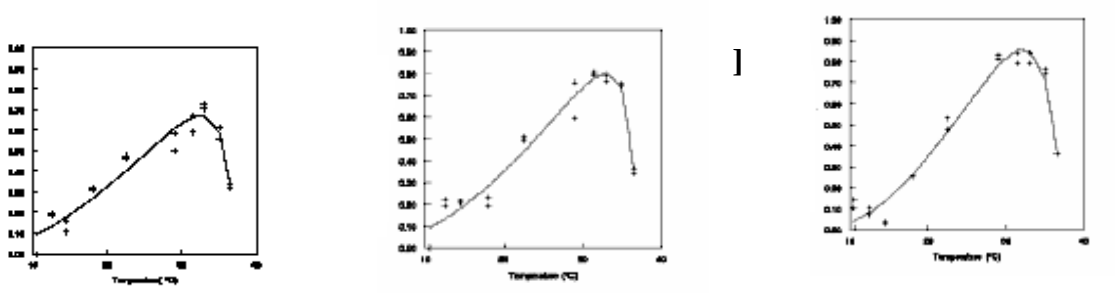
experiment has also been performed at low temperatures (such as 5°C; 6,5°C and 8,5°C) but the colonies didn't grow. It is difficult to determine the Tmin value because there are few points under 10°C. For the maximum temperature the experiment has been stopped at 36,5°C. By extrapolation the model gives a maximum temperature inferior to 40°C.



a **b** **c**

Figure 1. Development (growth rate) of *Aspergillus flavus* under different temperature conditions (strains: a- LABO 10038, b-MUCL 18903, c-NRRL 6550)

The linear regression coefficient is not very good for the value of Tmin (for the three strains the r^2 range between 0.93 – 0.96). If we had been modeling the effect of temperature on the growth rate between Tmin and Topt using a "growing" model type we could have demonstrated that for the three strains of *Aspergillus* the growth coefficient would be around the value 1.

The Rosso model has been elaborated on the bacteria cultures base. If these would have been treated by a "growing" type model, their coefficient would be near 2. It seems that the Rosso model is not appropriate to present the effect of temperature on the growth of *A. flavus*. The determination of Tmin using this model is difficult. For Tmax the experiment must be repeated using more temperatures between 36°C and 40°C.

2.2. Molecular study

Preliminary studies on *A. flavus* allowed the discovery of an ortologue of *fluG* gene to *A. nidulans* with a length of 214pb [11] after PCR amplification using the primers bellow.

The purpose was to find an ortologue of *fluG* gene for the three strains. The strategy is to work with specific primers of the gene *fluG* at *A. nidulans* (GenBank accession number L27817; Lee & Adams, 1994). Specific primers (named FluG1 to FluG6) of the gene *fluG* (3960bp) have been designed. For the *A. flavus* DNA amplification with four pairs of primers not separated by an intron (FluG1-FluG2, FluG1-FluG4, FluG3-FluG4, FluG5-FluG6) have been used and the chosen annealing temperature was 45°C (Rollet, 2004).

PCR products were separated by gel electrophoresis and their size have been determined (Figure 2). A certain number of gene fragments have been amplified, for each pair of primers used. For the couple of primers FluG3-FluG4, we have managed to obtain an amplified fragment which corresponds to a certain length for the toxic strains MUCL 18903 and LABO 10038. For the other pairs of primers more unspecific fragments were amplified without obtaining yet an amplicon. The length of the fragment amplified with the FluG3 and FluG4 primers is between 200 and 400bp, excepting the case of nontoxic strain NRRL 6550. For the MUCL 18903 strain (toxinogenic control strain), the amplified fragment is about 214bp.

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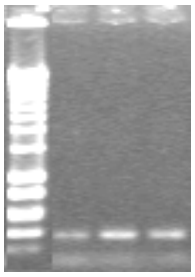

Figure 2. Electrophoresis of the PCR products amplified with *fluG* primers (FluG3 and FluG4). Lane 1, Smart Ladder; lane 2, LABO 10038; lane 3, MUCL 18903; lane 4, NRRL 6550

The results show that MUCL 18903 has an ortologue of the *fluG* gene. The strain LABO 10038 we found a completely different fragment. The exact length indicated by sequencing confirms the fact that the fragment obtained for MUCL 18903 is about 214 bp (figure 3), in the same time the fragment for LABO 10038 has 287 bp.

AF: SLIAQYPEVDFVLLHSSYPYTREAGYLACVYPNVYLDLGEVFPMVSRDAQESILR**D**SL**D**IVP**T**TRLLWST
FG: SLIAQYPEVDFVLLHSSYPYTREAGYLACVYPNVYLDLGEVFPMVSRDAQESILR**E**SL**E**IVP**S**TRLLWST

Figure 3. Comparison between the aminoacids sequence of the amplified fragment (AF) of *A. flavus* toxinogenic strain MUCL 18903 and the FluG aminoacids sequence (FG) of *A. nidulans*

The sequencing of the amplified fragment performed by Genome Express Society allowed the identification of a small part from the protein sequence which corresponds to the FluG protein from *A. nidulans*. Only three amino-acids from the protein sequence which corresponds to the amplified nucleic sequence at *A. flavus*, are different from the homologue protein sequence of the gene *fluG* at *A. nidulans* (the differences can only have minor consequences to the structure of the associated protein). If we find an ortologue of the gene *fluG* at MUCL 18903 this means that the results agree with the hypothesis that the aflatoxin and sterigmatocystin biosynthesis have the same biosynthesis pathway (Brown *& colab*., 1996a; Keller & Hohn, 1997)[1,6]. For the toxinogenic strain NRRL 6550 an ortologue of the *fluG* gene has not yet been identified so we can presume that the gene is not expressed.

The results obtained for the toxinogenic strain LABO 10038 and non toxinogenic strain NRRL 6550 are not enough conclusive to demonstrate the presence of an ortologue gene. It is possible that the primers used cover a little conserved region of the gene *fluG* at *A. nidulans.* If the gene *fluG* exists in the *A. flavus* genome, it is possible that the sequence could differ from a strain to another and because of the evolution of species or the environmental conditions this is not identical to the gene *fluG* at *A. nidulans*.

3.Conclusions

In the food chain, from the field to the consumer plate, molds can develop very easily and start production of aflatoxins especially in humid environments. Micotoxins are dangerous to human health but it is very difficult to completely eliminate them without harming the food's concentration in nutrients. The development and toxicity of these molds must be inhibited.

From the predictive microbiology point of view can be useful to obtain a double model for the growth and toxinogenesis for molds. Useful data can be obtained by studying the expression levels of micotoxins genes in various environmental conditions (RT-RealTime PCR). Also, finding an ortologue of *fluP* gene from *A.parasiticus* can complete the knowledge on the toxinogenesis mechanism.

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