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Interaction of Wild Strains of *Aspergilla* with A*spergillus parasiticus* ATCC15517 on Aflatoxins Production

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Abstract: Aflatoxins are secondary metabolites produced by some competent mould strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. These compounds have been extensively studied concerning its toxicity for animals and humans; they are able to induce liver cancer and caused a large range of adverse effects on living organisms. Aflatoxins are found as natural contaminants of food and feed; the main line of the strategy to control them is based on the prevention of the mould growth in raw vegetable or during its storage and monitoring of each crop batch. Moulds growth is conditioned by many ecological factors, including biotic one's. Hazard characterization models for Aflatoxins in crops must take in consideration the biotic interaction that moulds establish between them on their growth development. The aim of this work is to study the effect of the biotic interaction of 14 different wild strains of *Aspergilla* (different species), with a competent strain (*Aspergillus parasiticus* ATCC 15517) using an *in vitro* production model. The laboratorial model concerns to a natural matrix (humidified cracked corn), in which each wild strain challenged the producer strain for Aflatoxins production. Cultures were incubated at 28ºC for 12 days and sampled at 8th and 12th. Aflatoxins detection and quantification was performed by HPLC using a procedure with a MRPL = 1 µg/kg. Results of those interactive cultures revealed both synergic and antagonist effects on the Aflatoxin biosynthesis. Productivity increases were particularly evident at 8th day of incubation with wild strains of *A. flavipes*

(+ 70.4 %), *A. versicolor* (+ 54.9 %) and *A. flavus* 3 (+ 62.6 %). Antagonist effects were found with *A. niger* (- 69.5%) , *A. fumigatus* (- 47.6 %) and *A. terreus* (- 47.6 %) at 12th day. The increasable effects were more evident at 8th of incubation and the decrease was more patent at the 12th day. Results show that the development of *Aspergilla* strains concomitantly with competent aflatoxins producer moulds has a significant influence on the natural biosynthesis pattern.

Keywords: Aflatoxins, Micotoxins, Biosynthesis, *Aspergillus parasiticus, synergism*.

Introduction

Aflatoxins B_1, B_2, G_1, and G_2 are biotoxins synthesized on appropriated ecological conditions by some competent mould strains belonging to the groups of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. The ability of competent *Aspergilla* for Aflatoxins production depends on the individual metabolic systems, particularly to the primary metabolism of lipids and specified enzymes (synthetases) able to produce the secondary metabolites [1].

These secondary metabolites have been extensively studied since the earlier sixties, concerning its toxicity for animals and humans. Aflatoxins, especially B1, may induce liver cancer and caused a large diversity of adverse effects on living organisms: mutagenic, terathogenic and carcinogenic activities [2- 4]. The most constant effect is the depression on the synthesis of proteins, antibodies included. The severity of the adverse effects is proportionate to the level/doses of the exposition.

Nowadays, Aflatoxins are the most frequent hazard referred in food imported to EC, according to the annual reports of Rapid Alert System for Food and Feed [5].

Aflatoxins are found as natural contaminants of a large range of food and feed: cereals and other crops, dry fruits, and milk (ruminant's metabolites Aflatoxins M1 and M2).

The main strategic line to control Aflatoxins is based on the prevention of the mould growth in raw vegetable or during vegetative development, harvest, storage and transportation, through a adequate monitoring system applied to each crop batch. Moulds growth is conditioned by many ecological factors, including physical-chemical factors and also biotic ones. Levels of aflatoxins production are affected by many abiotic parameters like temperature, water availability, pH, osmotic pressure, oxi/reduction potential and chemical nature of nutrients.

The ecological conditions that are favourable to Aflatoxins biosynthesis are also appropriated for the growth of all the concomitant moulds that colonize a specific crop. Under this perspective it is important to understand in what extension these developments of the mycobiota may interfere with the normal biosynthesis of Aflatoxins (biotic factors).

The full knowledge of biosynthesis pathway will only became better understood when interactive and multi-factorial studies were performed. The aim of this work was to study the influence of biotic interaction, using an *in vitro* model, where 14 different wild strains *Aspergilla* challenged an Aflatoxins producer strain of *A. parasiticus* (ATCC 15517).

Materials and Methods

Strains

A. parasiticus ATCC 15517 (aflatoxins producer strain) and 14 wild *Aspergilla* strains commonly found in crops (won laboratory collection): *A. candidus, A. clavatus, A. flavipes, A. restritus, A. niger, A. versicolor, A. ochraceus, A. glaucus, A. terreus, A. fumigatus and A. flavus* (5 isolates). *A. flavus* isolates were previous tested for their ability to synthesize Aflatoxins and revealed negative. Moulds colonies were maintained into Czapek agar (OXOID CM. 549), incubated at 25° C for 8 days [3].

"In vitro" aflatoxin's production model

Each culture for aflatoxin production was performed challenging individually the competent *A. parasiticus* reference strain. Each assay were carried out in four Erlenmeyer flasks containing 50 g of sterilised cracked corn each, added of distilled water for an a_W adjustment to 0.98 [6].

Autoclaved substrate was inoculated separately with spore suspensions:

a) Testimony were inoculated with 2 ml of spore suspension of *A. parasiticus* ATCC 15517, diluted to half; Spores density were estimated using an opacity gradient equivalent to 0.5 Macfarland.

b) Interactive cultures of the two strains (*A. parasiticus* ATCC 15517 more each of the other *Aspergilla* isolates) were inoculated with 1 ml of each spore suspension.

The flasks that were inoculated with the testimony and with the mixed strains were manually shaken daily during 5 min to obtain an adequate homogeneity. Incubations were performed at 28°C for 8 and 12 days for two series of flask culture, respectively [7].

Extraction and Immunoaffinity Column Chromatography

Extraction and immunoafinity column clean step was performed according to the method described by Stroka and Anklam, 2000 [8].

The samples were extracted with acetonitrile-water solution (85/15) (V/V). The extracted was filtered and diluted (5 ml) with phosphate buffer saline (PBS) (95 ml). Filtrate was passed through the immunoaffinity column (Afla B G.1003, VICAM) and AFS were eluted with 1.25 ml and washed with 1.75 ml of water. Eluate was collected and directly used in the HPLC system.

Aflatoxin quantification (HPLC)

Determination of aflatoxins levels in samples extracts was carried out by isocratic reverse-phase liquid chromatography (HPLC) using a LiChrospher 100 RP-18 (5 μm column 25 x 4.6 mm i.d.) EcoPack (Merck, Portugal), with post column derivatization involving bromination, with pyridinum hydrobromide perbromide (PBPB) (Sigma P- 3179) (Quimica S. A., Spain) and with fluorescence detector Merck Hitachi; excitation and emission wavelengths were 360 nm and 420 nm, respectively.

The mobile phase was water-acetonitrile-methanol solution (6/2/3) (V/V/V), and the flow rates were 1.00 ml/min for mobile phase and 0.30 ml/min for reagent PBPB. The MRLP was 1 μg / kg.

Results and Discussion

A. parasiticus ATCC 15517 was used as producer strain (testimony). The global productivity of Aflatoxins B1, B2, G1 and G2 was 25.8 mg/kg at the 8th day and 42.0 mg/kg at the 12th day (Table 1).

Table 1. Data analysis about the productivity of aflatoxins B1, B2, G1 and G2 by *A. parasiticus* (ATCC 15517) in cracked corn, at the 8th and 12th day of incubation.

Culture of *A. parasiticus*	**8th day**	**12th day**
No. assays	14	14
Global Productivity (mg/kg)	25.8	42.0
Standard deviation (SD)	1.42	3.6
Variance	2.02	12.8
Confidence Level (p= 0.05)	0.75	1.88

The used model for *in vitro* production revealed a higher level of Aflatoxins at the 12th day of incubation, 68.0 mg of Aflatoxins / kg of humidified cracked corn (Table 3). The specificity of the interaction to a synergic or an antagonistic effect was confirmed in both times of incubation, with the exception of *A. flavus* 1 and *A. glaucus* that revealed to be synergic at 8th of incubation and slightly antagonist at 12th (Table 4). This may signify that there is a specific tendency in the interaction effects of *Aspergilla* strains when they have a concomitant development with a competent Aflatoxin´s producer one, in a particular matrix.

Tendency the antagonistic effects seems to be more evident at the 12th day of incubation and the synergic effect was higher, in percentage terms, at the 8th day of incubation (Tables 2 and 3).

The results of the interaction effects of the wild A*spergillus* strains concerning the production level of each of the four aflatoxins by *A. parasiticus* (ATCC 15517), showed special differences with Aflatoxin B1, that had always the highest level, and G2, the lowest one (Tables 2 and 3).

Synergic activities were detected with strains of *A. candidus, A. clavatus, A. flavus , A. flavipes A. versicolor* and *A. restritus,* at both period of incubation. The increase of production may raise more than 50% of the testimony productivity (3 strains) (Table 3). The strains that revealed to have higher synergism effects were: *A. flavus* 3 (+ 62.6%), *A. flavipes* (+70.4%) and *A. versicolor* (+ 54.9 %), at the first incubation period. The intermediate metabolic mechanism that may explain this behaviour is not completely elucidated, although it may be a consequence of some possible intermediated metabolites that can be used by the competent strain to synthesize Aflatoxins. The synergism may result of the capacity of the non-toxigenic strains to produce ethylene, or acetate, metabolite that is a useful precursor to the biogenesis chain of aflatoxins [9]. Badii *et al*. [10] related that precursor metabolites of the aflatoxins biosynthesis, may justify the synergism between interactive cultures. Another possibility is related to the fact that the non toxigenic strains can also achieve the production of sterigmatocystin and 0-metil-sterigmatocystin, chemical precursors of the aflatoxins [11].

Table 2. Productivity of Aflatoxins B1, B2, G1 and G2 in cracked corn, at the 8th day of incubation and respective culture.

Cultures	Productivity* (mg/kg)				
	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	**Total**
A. parasiticus (Ap)	**10.1**	**8.2**	**3.5**	**3.6**	**25.4**
Ap + A. candidus	12.0	10.0	8.0	6.0	**36.0**
Ap + A. clavatus	12.0	10.0	8.0	6.0	**36.0**
Ap + A. flavipes	18.0	12.0	8.0	6.0	**44.0**
Ap + A. flavus 1	10.0	9.0	9.0	6.0	**34.0**
Ap + A. flavus 2	12.0	10.0	9.0	6.0	**37.0**
Ap + A. flavus 3	18.0	10.0	8.0	6.0	**42.0**
Ap + A. flavus 4	12.0	10.0	6.0	5.4	**33.4**
Ap + A. flavus 5	16.0	10.0	6.0	4.0	**36.0**
Ap +A. fumigatus	6.0	6.0	3.2	2.0	**17.2**
Ap + A. glaucus	12.0	10.0	6.0	6.0	**34.0**
Ap + A. niger	3.0	3.0	1.0	1.0	**8.0**
Ap + A. restrictus	12.0	12.0	6.0	6.0	**36.0**
Ap + A. terreus	6.0	6.0	3.2	2.0	**17.2**
Ap+ A. versicolor	12.0	12.0	10.0	6.0	**40.0**

* Average of 2 assays

Investigations [11] have demonstrated that when these percursors are added to a culture media, in which a toxigenic strain is developing, may increase 3 to 25 times the level of productivity when referred to a single testimony strain. Synergism may also result of a better efficiency of the competent strain to use the nutrients of the matrix when its metabolism are complementary of the challenged strain; the non toxinogenic strains may have the hability to metabolize each of the nutrients by a different pathway having, for example, a more acentuated proteolitic activity than the toxinogenic strain.

The antagonistic effects were especially evident in the interactive cultures with *A. terreus* (- 47.6%), *A. fumigatus* (- 47.6%) and *A. niger* (- 69.5%), more marked at the 12th of incubation (Table 4). The decrease of the production was more noted on the fraction Aflatoxin B1 (Table 3).

The explanations for the antagonistic effects are not yet completely elucidated but it may be explained by the higher capacity of the challenger strain to more quickly metabolize essential nutrients of the matrix of to promote biodegradation of the previous formed Aflatoxins. This second hypothesis is in accordance with the fact that antagonist effects were more evident with the longer period of incubation (12 days). The more sustained explanation evocates the ability of some Aspergilla, especially *A. niger*, to produce organic acids, like citric acid, that allows to a precocious pH decreasing of the substrate (pH= 3.1 to 3.7), promoting, through this via, the inhibition of growth of the competent Aflatoxins producer strain [12].

Table 3. Productivity of Aflatoxins B1, B2, G1 and G2 in cracked corn, at the 12th day of incubation and respective culture.

Cultures	Productivity* (mg/kg)				
	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	**Total**
A. parasiticus (Ap)	**18.0**	**13.3**	**7.0**	**3.7**	**42.0**
Ap + A. candidus	18.0	16.0	10.0	10.0	**54.0**
Ap + A. clavatus	18.0	18.0	12.0	12.0	**60.0**
Ap + A. flavipes	30.0	20.0	12.0	6.0	**68.0**
Ap + A. flavus 1	18.0	6.0	9.0	6.0	**39.0**
Ap + A. flavus 2	20.0	10.0	9.0	6.0	**45.0**
Ap + A. flavus 3	24.0	10.0	12.0	6.0	**52.0**
Ap + A. flavus 4	20.0	18.0	12.0	6.0	**56.0**
Ap + A. flavus 5	20.0	16.0	9.0	8.0	**53.0**
Ap +A. fumigatus	8.0	8.0	4.0	2.0	**22.0**
Ap + A. glaucus	16.0	12.0	4.0	3.0	**35.0**
Ap + A. niger	6.0	4.0	1.4	1.4	**12.8**
Ap + A. restrictus	20.0	16.0	10.0	6.0	**52.0**
Ap + A. terreus	8.0	8.0	4.0	2.0	**22.0**
Ap+ A. versicolor	18.0	16.0	10.0	10.0	**54.0**

*Average of 2 assays

Table 4. Productivity deviation ratio (%) by *Aspergillus* strains relative to the testimony (*A. parasiticus*) global productivity of aflatoxins B1, B2, G1 and G2.

Cultures	Productivity deviation ratio (%) by *Aspergillus* strains relative to the testimony		Interactions
	8th day	**12th day**	
Ap + A. candidus	39.4	28.6	Synergic
Ap + A. clavatus	39.4	42.9	Synergic
Ap + A. flavipes	70.4	62.0	Synergic
Ap + A. flavus 1	31.6	-7.1	Synergic/ Antagonist
Ap + A. flavus 2	43.3	7.2	Synergic
Ap + A. flavus 3	62.6	23.9	Synergic
Ap + A. flavus 4	29.3	33.4	Synergic
Ap + A. flavus 5	39.4	26.2	Synergic
Ap +A. fumigatus	-33.4	-47.6	Antagonist
Ap + A. glaucus	31.6	-16.6	Synergic/Antagonist
Ap + A. niger	-69.0	-69.5	Antagonist
Ap + A. restrictus	39.4	23.9	Synergic
Ap + A. terreus	-33.4	-47.6	Antagonist
Ap+ A. versicolor	54.9	28.6	Synergic

The antagonistic effects of *A. terreus* are probably related to the fast utilization of nutrients, instead of aflatoxins biodegradative capacity, since the aflatoxins B1, B2, G1 and G2 productivity decrease is proportional. Concernig to *A. fumigatus*, the substrate conditions were unfavorable for its development. For *A. fumigatus* the optimal in temperature growing conditions is superior to 25º C. The present study shows that the biosynthesis pathway for Aflatoxins is clearly influenced by the interaction of other moulds that may co-colonize the crops were Aflatoxins production occurs. Co-existence and growth of mycobiota in a particular substrate allows to different results on level of Aflatoxins production capacity of the competent strains. Taking this in consideration, risk assessors must attend to biotic interactions when they develop models for the characterization of this hazard.

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