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Interaction of strains of non-toxigenic *Aspergillus flavus* with *Aspergillus parasiticus* on aflatoxin production

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Interacções de estirpes de *Aspergillus flavus* não-toxígenas com *Aspergillus parasiticus* na produção de aflatoxinas

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SUMMARY

Aflatoxins production is affected by abiotic, biotic and genetic parameters. Eugenesic and dysgenetic condition for aflatoxinogenesis are relatively well studied, such as: the influence of temperature, pH, Aw, Oxigen tension, osmotic pression, organic and inorganic nutrients, substance with fungistatic effects: however there are relatively few knowledge about endogenous regulator mechanisms, the kinetic of the anabolism and the interaction between the productive moulds and remaining microflora holding in eutrophying substrates. The main objective of this study is to evaluate biotic interaction between five indigenous strains of *A. flavus*, confirmed non-toxigenic, and a productive strain (A. parasiticus ATCC 15 517), cultured simultaneously on two substrates: one natural (cracked corn) and a synthetic one, modified Czapeck-Dox broth. Aflatoxins quantifications were performed by HPLC at 8th and 12th days. Results of those interactive cultures showed that all strains were synergic, increasing aflatoxins production in a range between 5.6 and 106.5% over the control values, with an exception registered on one of the strains cultured in cracked corn at the 12th day, where the production decreased (-7.6%).

UNITERMS: Plant interaction; Aflatoxins; Vegetal production; *Aspergillus flavus*; *Aspergillus parasiticus*.

INTRODUCTION

Aspergillus flavus and *A. parasiticus*, producers of aflatoxins (AFs), are closely related fungi that contaminate seeds and plant debris of many crops in the field, during harvest, in storage, and during processing. *A. flavus* seems adapted to the aerial and foliar environment being dominant in corn, cottonseed and tree nuts whereas *A. parasiticus* appears to be adapted to a soil (peanuts). Many strains of *A. flavus* are not toxigenic, so the presence of mouldiness is not, of itself, indicative of toxin production[2].

The capacity for aflatoxins production depends on the individual metabolic systems, essential to the primary metabolism of lipids and specified enzymes (synthetases) able to produce this secondary metabolite.

Levels of aflatoxins production are affected by many abiotic parameters like temperature, water activity, pH, osmotic pressure, substrate nature and also by biotic factors. Many of these parameters have been investigated, most of the time, separately, but it must be realised that they all interact in natural conditions. The full knowledge of biosynthesis pathway will only became better understood when interactive and multi-factorial studies were performed. Based on its potent genotoxicity and hepatotoxicity and its widespread occurrence in food commodities, aflatoxin B_1 is most a significant initiator and promoter of human primary hepatocellular carcinoma[9,11]. Aflatoxin B_1 has induced liver cancer in all the species of laboratory animals tested[25] and is an extremely potent carcinogen to the rats and rainbow trout. According to Diener *et al.*[5], toxinogenic strains of *Aspergillus flavus* usually produce higher levels of aflatoxin B_1 and aflatoxin B_2, whereas with *A. parasiticus* more equitative amounts of aflatoxins B_1, B_2, G_1, and G_2 are obtained.

The main objective of this study was to evaluate biotic interactions between non-toxigenic wild strains of *A. flavus*, and a toxigenic strain (*Aspergillus parasiticus* ATCC 15517), cultured on two substrates: one natural (cracked corn) and another available in Czapeck Dox Broth (Oxoid), modified by Martins *et al.*[15].

MATERIAL AND METHOD

Strains: A. parasiticus ATCC 15517 and non-toxigenic *A. flavus* (five wild strains) obtained from mixed feed were used to aflatoxin production. Colonies of *A. flavus* (wild) were picked to Czapek agar (Difco) plates, and incubated at 25°C for 8 days. These isolates were identified, considering macroscopic and microscopic morphological aspects, compared to descriptions given by Raper and Fennel[22], and Domsch *et al* [6], and previously confirmed as non-toxigenic[18].

***In vitro* aflatoxin production:** The studies on aflatoxin production by *A. parasiticus* ATCC 15517, were carried out in duplicate on Erlenmeyer flasks containing the following substrates:

a) 50 ml of Czapeck Dox Broth base (MCD) (Oxoid) modified with adding: 20 g of Saccharose (Merck) (instead of glucose); 20 g of Yeast extract (Difco); 0.005 g Zinc Sulphate (Merck); 4 g Citric acid (Merck); 1,000 ml of bi-distilled water with 30% of corn infusion[8,15];

b) 50 g of sterilised cracked corn, adding 20 ml of distilled water and adjusting Aw to 0.98[18].

Autoclaved substrates were inoculated separately with spore suspensions: Blanks were inoculated with 2 ml of spore suspension of *A. parasiticus* ATCC 15517; Interactive cultures (*A. parasiticus* ATCC 15517 and each of the non-toxigenic *A. flavus*) were inoculated with 1 ml of each spore suspension according the following procedure: 5 ml of sterile distilled water was added to each slant of five days old culture and gently scraping the agar surface to give a turbid suspension, till an absorption density between 0.300 and 0.400 at 450 nm wavelength on Spectrophotometer (Model Junior III, Coleman 6/8). Two ml of this suspension were added to the MCD broth and to the cracked corn.

Inoculated flasks were shaken daily for the first three days. Incubations for *in vitro* AFs production were performed at 28°C for 8 days and another series was incubated at the same temperature for 12 days.

Aflatoxin quantification by High Pressure Liquid Chromatografhy (HPLC):

The quantification of aflatoxins (AFs) was processed according to the method described by van Egmond[24]. The aflatoxins were extracted with chloroform, filtered and purified of an aliquot portion over a Florisil cartridge (Sep-Pak, Waters), subsequently followed by a C18 cartridge (Sep-Pak; Waters). Determination of aflatoxins (levels were 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 derivatisation according to Garner *et al.*[7], involving bromination, with pyridinum hydrobromide perbromide (PBPB) (Sigma P- 3179). The detection limit was 0.001 mg/kg. Standard Aflatoxins B_1, B_2, G_1 and G_2 were purchased from Sigma (A-6636).

Correlation fungal biomass/aflatoxin production: Fungal biomass of each culture flasks (blank and interactive cultures) in MCD, at 8th and 12th days, were removed to a Petri disk, dehydrated at 120°C for 15 min and then weighted, to correlate with production (productivity mg of AFs/g biomass).

RESULTS

Concerning the five wild strains of *A. flavus* previously tested and proved to be non-toxigenic, it was verified, in all cases, that they were synergic with productive strain (Tab. 1, 2, and 4). Synergism was also observed in mycelial development, since biomasses of mixed colonies were higher than the reference culture. It was evident that there was a relative constant average concerning Aflatoxins production per unity of fungal biomass (Tab. 3).

Aflatoxins global production, in both substrates, was higher in mixed cultures than in the control one (*A. parasiticus* ATCC 15517), cultured individually. The wild *A. flavus* 2 had a remarkable synergic activity, with the competent strain, in MCD, presenting an increase of

Table 1

Production of Aflatoxins (AFs) and comparative deviation ratio, at 8 days of incubation in MCB (mg/kg). Lisboa, 1998/1999.

	Aflatoxins (mg / Kg)				
MOULD'S CULTURES	B_1	B_2	G_1	G_2	Total
A. parasiticus (ATCC 15517)	8.6	3.6	3.5	3.4	19.1
A. parasiticus + *A. flavus* 1	12.0	6.0	6.0	6.0	30.0
(Deviation ratio)	(+3.4)	(+2.4)	(+2.5)	(+2.6)	(+10.9)
A. parasiticus + *A. flavus* 2	12.0	8.0	6.0	6.0	32
(Deviation ratio)	(+3.4)	(+4.4)	(+2.5)	(+2.6)	(+12.9)
A. parasiticus + *A. flavus* 3	12.0	4.0	5.4	4	25.4
(Deviation ratio)	(+3.4)	(+0.4)	(+1.9)	(+0.6)	(+6.3)
A. parasiticus + *A. flavus* 4	9.0	4.0	3.6	3.6	20.2
(Deviation ratio)	(+0.4)	(+0.4)	(+0.1)	(+0.2)	(+1.1)
A. parasiticus + *A. flavus* 5	12.0	6.0	4.0	6.0	28.0
(Deviation ratio)	(+3.4)	(+2.4)	(+0.5)	(+2.6)	(8.9)

Table 2

Production of Aflatoxins (AFs) and comparative deviation ratio at 12 days of incubation in MCB (mg/kg). Lisboa, 1998/1999.

	Aflatoxins (mg/ Kg)				
MOULD'S CULTURES	B_1	B_2	G_1	G_2	Total
A. parasiticus (ATCC 15517)	13.4	3.9	3.9	3.9	21.3
A. parasiticus + *A. flavus* 1	16.0	8.0	8.0	8.0	40.0
(Deviation ratio)	(+2.6)	(+4.1)	(+4.1)	(+4.1)	(+18.7)
A. parasiticus + *A. flavus* 2	18.0	10.0	8.0	8.0	44
(Deviation ratio)	(+4.6)	(+6.1)	(+4.1)	(+4.1)	(+22.7)
A. parasiticus + *A. flavus* 3	18.0	8.0	6.0	5.4	37.4
(Deviation ratio)	(+4.6)	(+4.1)	(+2.1)	(+1.5)	(+16.1)
A. parasiticus + *A. flavus* 4	12.0	6.0	4.0	3.6	25.6
(Deviation ratio)	(+1.4)	(+2.1)	(+0.1)	(+0.3)	(+4.3)
A. parasiticus + *A. flavus* 5	16.0	6.0	5.4	14.0	32.8
(Deviation ratio)	(+2.6)	(+2.1)	(+1.5)	(+10.1)	(+11.5)

Table 3

Productivity ratio of Aflatoxins (AFs) in MCB at 8 and 12 days of incubation in function of fungal biomasses. Lisboa, 1998/1999.

	Aflatoxins mg /Kg	g of Fungal Biomass		Productivity (*)	
MOULD'S CULTURES	Total	T 8	T 12	T8	T12
A. parasiticus (ATCC 15517)	19.1	172.8	268.4	0.11	0.079
A. parasiticus + *A. flavus* 1	30.0	296.0	334	0.10	0.11
A. parasiticus + *A. flavus* 2	32	282.0	326	0.11	0.13
A. parasiticus + *A. flavus* 3	25.4	276.0	321.4	0.09	0.11
A. parasiticus + *A. flavus* 4	20.2	254.0	300.2	0.07	0.08
A. parasiticus + *A. flavus* 5	28.0	234.0	294	0.11	0.11

Legend – (*) – mg AFs/g fungal biomass; T8 – Time of incubation – 8 days (25°C); T12 – Time of incubation – 12 days (25°C).

Table 4

Production of Aflatoxins (AFs) in cracked corn at 8th day of incubation (mg/kg). Lisboa, 1998/1999.

	Aflatoxins (mg/ Kg)				
MOULD'S CULTURES	B_1	B_2	G_1	G_2	Total
A. parasiticus (ATCC 15517)	8.6	3.6	3.5	3.4	19.1
	0.52	0.44	0.183	0.183	1.327
A. parasiticus + *A. flavus* 1	10.0	9.0	9.0	6.0	34
(Deviation ratio)	(+1.4)	(+5.4)	(+5.5)	(+2.6)	(+14.9)
A. parasiticus + *A. flavus* 2	12.0	10.0	9.0	6.0	37
(Deviation ratio)	(+3.4)	(+6.4)	(+5.5)	(+2.6)	(+17.9)
A. parasiticus + *A. flavus* 3	18.0	10.0	8.0	6.0	42.0
(Deviation ratio)	(+9.4)	(+6.4)	(+4.5)	(+2.6)	(+22.9)
A. parasiticus + *A. flavus* 4	12.0	10.0	6.0	5.4	33.4
(Deviation ratio)	(+3.4)	(+6.4)	(+2.5)	(+2)	(+14.3)
A. parasiticus + *A. flavus* 5	16.0	10.0	6.0	4.0	36.0
(Deviation ratio)	(+7.4)	(+6.4)	(+2.5)	(+0.6)	(+16.9)

production that was 106.5% higher than the average of the control culture, at the 12th day (Tab. 2).

In a general appreciation, the synergism observed on those mixed cultures increases between 5.6% and 106.5% over the average control values (Tab. 1, 2, 4 and 5); with an exception registered with *A. flavus* 1, 2 and 3 strains cultured on cracked corn at 12th day, which the production decreased considerably (-7.6% and -3.6%) (Tab. 5).

DISCUSSION AND CONCLUSIONS

A. flavus are natural contaminants of many feeds and raw materials for human and animal consumption. On a survey conducted during four years, Martins[12] found *A. flavus* in about 70.0% of feeds (1,103 samples), and in 42.6% of raw materials. In Australia, Bryden *et al.*[4] found prevalence over 80% of *A. flavus* on those materials. Martins[18] in a study about *in vitro* aflatoxin production by *Aspergillus flavus* (114 strains) isolated from raw materials and mixed feed found 60.5% toxigenic strains and the range of the aflatoxin B_1 was 0.004 to 391.9 mg/kg.

Table 5

Production of Aflatoxins (AFs) in cracked corn in five interactive cultures at 12th day of incubation (mg/ kg). Lisboa, 1998/1999.

	Aflatoxins (mg/ Kg)				
MOULD'S CULTURES	B_1	B_2	G_1	G_2	Total
A. parasiticus (ATCC 15517)	17.6	13.6	7.2	3.8	42.2
A. parasiticus + *A. flavus* 1	18.0	6.0	9.0	6.0	39.0
(Deviation ratio)	(0.4)	(-7.6)	(+1.8)	(2.2)	(-3.2)
A. parasiticus + *A. flavus* 2	20	10.0	9.0	6.0	45.0
(Deviation ratio)	(+2.4)	(-3.6)	(+1.8)	(+2.2)	(2.8)
A. parasiticus + *A. flavus* 3	24.0	10.0	12.0	6.0	52.0
(Deviation ratio)	(+6.4)	(-3.6)	(+4.8)	(+2.2)	(+9.8)
A. parasiticus + *A. flavus* 4	20.0	18.0	12.0	6.0	56.0
(Deviation ratio)	(+2.4)	(+4.4)	(+4.8)	(+2.2)	(+13.8)
A. parasiticus + *A. flavus* 5	20.0	16.0	9.0	8.0	53.0
(Deviation ratio)	(+2.4)	(+2.4)	(+1.8)	(+4.2)	(+10.8)

The present study shows that indigenous strains of *A. flavus* previously tested and classified as non-toxigenic can be synergetic with toxigenic strains. This synergism may be due to the capacity of the non-toxigenic strains to produce ethylene and to the fact that this metabolite, being a precursor of acetate, is useful to the biogenesis chain of aflatoxins[23]. Badii *et al.*[1] related that precursor metabolites of aflatoxin biosynthesis may justify the synergism between interactive cultures. Another possibility concerns to the fact that the non toxigenic strains can also achieve the production of sterigmatocystin and 0-metil-sterigmatocystin, chemical precursors of the aflatoxins Pro *et al.*[21]. The same authors showed that the addition of these precursors to a culture medium, where the development of a toxigenic strain takes place, increases the production levels of 3 to 25 times. Nevertheless, Brown *et al.*[3] verified that in natural conditions the addition of telluric non-toxigenic *A. flavus* to corn, during the harvest, prevented the posterior colonisation by toxigenic strains, and even when those strains develop on the maize, during the storage, they are capable of biodegrading the previously formed aflatoxins. The assimilation by mycelia, in the vegetative phase of the development of non-toxigenic strains, seems to justify detoxification process, of the toxin in the subtracts[20].

Martins *et al.*[19] studied the role of *Fusarium moniliforme* on aflatoxins production, and verified that the global aflatoxins productivity decreased about 30% on MCD, and 26% on cracked corn. In another interactive studies with *A. terreus*, *A. niger* and with *Mucorales* the *in vitro* aflatoxin production also decreased[5,14,17]. According to Jarvis[10], *Rhizopus oryzae* strains have biological capacity to degrade the aflatoxins.

Other interactions performed with *Saccharomyces cerevisae* ATCC 97 631[13] and with *Penicillium* spp[16] showed increase of the aflatoxin productivity.

The present study confirms synergic interaction of mould development and a potential increase of aflatoxin productivity. In the evaluation of the toxigenic capacity of the different strains of *A. flavus*, the interaction with the other mycoflora components of the substrate should always be taken in consideration. Otherwise the intervenue on the aflatoxinogenesis mechanism can be under or over evaluated.

RESUMO

A produção de aflatoxinas é afetada por parâmetros abióticos, bióticos e genéticos. As condições eugenésicas e disgenésicas da aflatoxinogênese estão relativamente bem estudadas, tal como as influências ecológicas (temperatura, pH, Aw, tensão de oxigênio, pressão osmótica, nutrientes e substâncias fungistáticas). Contudo, é muito escasso o conhecimento relativo aos mecanismos reguladores endógenos, à cinética do anabolismo e à interação dos fungos produtores com a restante microflora presente em substratos eutrofizantes. O principal objetivo deste estudo é avaliar a interação de cinco estirpes indígenas de *A. flavus*, comprovadamente atoxígenas, com uma geneticamente apta (*A. parasiticus* ATCC 15517), cultivadas simultaneamente em dois substratos: um natural (milho triturado) e outro sintético (Caldo de Czapeck-Dox Modificado). A quantificação das aflatoxinas foi efetuada no 8º e 12º dias de incubação, por Cromatografia Líquida de Alta Resolução (HPLC). Os resultados demonstraram que todas as estirpes foram sinérgicas, aumentando o rendimento da produção entre 5,6 e 106,5%, quando comparados com os valores das testemunhas.

UNITERMOS: Interação de plantas; Aflatoxinas; Produção vegetal; *Aspergillus flavus*; *Aspergillus parasiticus*.

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