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Aflatoxin production on cereals, oil seeds and some organic fractions extracted from sunflower (**)

SUMMARY. — Two strains of *Aspergillus flavus* develop on cereals (wheat and maize) and on oil seeds (arachida, groundnuts, sunflower) in a different way. The production of aflatoxins is different both qualitatively and quantitatively. The fungal growth is not strictly connected with the production of toxins. The highest production of aflatoxins was observed when both strains were grown on sunflower seeds. The organic fractions separated from the seed influence the aflatoxin production.

RIASSUNTO. — Due differenti ceppi di *Aspergillus flavus* si sviluppano in diversa maniera su cereali (grano e mais) e su semi oleosi (arachide, nocciola, girasole). Anche la produzione di aflatossine è differente qualitativamente e quantitativamente. Lo sviluppo del fungo non è strettamente connesso con la produzione delle tossine. La maggiore produzione di aflatossine si è rilevata quando entrambi i funghi si sviluppavano su semi di girasole. Diverse frazioni organiche estratte influenzano in modo differente la produzione di aflatossine.

INTRODUCTION

For some years we have studied the production of aflatoxins both "in vitro" and "in vivo" conditions [1-3]. Many strains of *Aspergillus flavus* produce aflatoxins in different manners and for this reason the mere presence of mouldness is not, in itself, an indicative datum of toxins production. The natural occurence of different aflatoxins can be described only when the initial inoculation with the mould spores and subsequent mould development with the production of aflatoxins are a natural sequence of events [4]. The *Aspergillus flavus* has the capability to grow on a wide range of agricultural crops particularly when the alimentary products are stored, for this reason this fungus is considered a

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storage fungus [5]. In the present paper we have analysed two strains of the fungus and their production of aflatoxins on different groups of crops including maize, arachids, wheat, groundnuts and sunflower to verify the differences among the assayed seeds. As on sunflower seeds the *A. flavus* produced the highest amount of aflatoxins we have also analysed the effect of different organic fractions of sunflower seeds on the production of aflatoxins.

Materials and methods

Culture conditions

The assayed strains of the fungus were *Aspergillus flavus* (ATCC 22548) and str. CF1 isolated in our Institute from wheat. Both strains of the fungus were kept on Czapek Dox medium (Difco) at 25°C.

The seeds used for our experiments were wheat (Manitoba), maize (Decalb 365), sunflower (Uniflor 70), groundnuts (Piemontesi) and arachids (Bombay). For each seed we verified the fungal microflora on the stored seeds to be later inoculated with fungal conidia of *Aspergillus flavus*.

As the seeds assayed were stored in silos at very low percentage of moisture the fungal microflora present was very scant and for this reason we used the seeds without sterilization considering that the *Aspergillus flavus* could easily grow on all seeds without any slackering because of interfering microflora.

The seeds were then moistened with sterile distilled water at a value that was suitable for the growth of the fungus; for each seed a different moisture value was used: wheat 18.5%, maize 21.7%, sunflower 11.0%, arachids 11.0%, groundnuts 11.0%.

50 grams of seeds were placed in a 250 Erlenmeyer flask at 30°C and then inoculated with 8×10^5 15 days old conidia of *A. flavus* produced on Czapek Dox agar. After different days of incubation two flasks for each kind of seeds were analysed and the fungal growth and aflatoxin production examined.

The detection of fungal growth was made by the diluition plate method: 10 grams of ground seeds (by Stomaker) were placed directly in 100 ml of saline solution (NaCl 0.9%). The inoculum was made by plating different diluitions of the suspension on mycological agar (Difco). Incubation was carried out at 25°C for 5 days.

In order to extract different organic compounds from sunflower kernels with no hull we used: N-hexane for 7 h at 30°C, for lipids (Lipid Fraction: LF); ethanol: water (50 : 50 v/v) for 30 minutes at room temperature, for carbohydrates (Carbohydrates Fraction: CF); the remaining seed was considered as protein fraction (PF).

The different fractions extracted to be used as a substrate for the fungus growth presented the following composition: hull 30%, carbohydrates fraction (CF) 10%, lipid fraction (LF) 40%, protein fraction (PF) 20%. The fungus was inoculated on the four extracted fractions thus artificially recon-

structing the seed. We also have inoculated three fractions only, excluding each time one of the four. This in order to assay the effect of the absence of every fraction on the aflatoxin production and the fungal growth.

Analysis of aflatoxins

After the growth of the fungus the seeds were omogenized by using Waring Blendor and extracted with chloroform and methanol (2 : 1 v/v) for 3 hours by Soxhlet. The extracts were filtered through phase separative paper (Whatman 1 PS) and concentrated on rotary evaporator. To purify the aflatoxins from the lipids present in the sample, a thin layer chromatography run was made on a layer of Stratocrom SI AP, Carlo Erba: N-hexane-ethylether-aceticacid (70 : 30 : 1.5 v/v/v).

The aflatoxins do not migrate and are thus totally recovered without any alteration. They were concentrated in a fixed volume of HPLC elution solvent and analysed according to Beebe and Takahashi [6]. As HPLC apparatus we used the Hewlett Packard Liquid Chromatograph model 1080 with 79850 A LC terminal and detector at 362 nm.

In order to verify wether the aflatoxins produced on different seeds were concentrated in the seed or in the conidia and mycelium of the fungus which completely covered their surface, we separated and collected the conidia and mycelium of *Aspergillus flavus* from the seeds infected with the fungus. We used only sunflower seeds because the production of toxins was the highest and thus the results were more easily verifiable.

100 grams of mouldered seeds were repeatedly (10 times) washed in distilled water and so the collected conidia and mycelium were concentrated by filtration through Millipore filters (0.45 μm) and dried at 80°C for 48 h. We measured the dry weight of the fungus produced and collected at different times and the corresponding production of aflatoxins according to the described method.

Results and discussion

The growth and the production of aflatoxins of the two strains of *Aspergillus flavus* on cereals and oil seeds is very different (Table 1). The strain ATCC in all cases grows better as compared with the strain CF1 and the growth of both is more evident on oil seeds as compared with cereals. The growth at the 8th day is clearly the most evident result considering that in all cases we only detected *Aspergillus flavus* in the seeds. On the 21st day instead we found in three cases the growth of the other fungal microflora (*Aspergillus niger* and *Penicillium cyclopium*).

Obviously this result can have influenced the growth of *Aspergillus flavus* interfering both with the growth and the production of aflatoxins. The production of the different toxins produced in the different seeds is evident and it appears

TABLE 1 — *Growth and production of aflatoxins of two different strains of Aspergillus flavus on different seeds after 8 and 21 days of incubation at 30°C.*

SEEDS		DAYS OF INCUBATION 8 growth (numb. colonies/gram)	8 aflatoxins (µg/gram)	21 growth (numb. colonies/gram)	21 aflatoxins (µg/gram)
ARACHIDS	strain ATCC	1131×10^4	0.092	40.8×10^6 *	98.48
	strain CF1	80×10^4	0.036	17.4×10^6 **	94.32
SUNFLOWER	strain ATCC	5320×10^4	0.14	64.8×10^6	41.60
	strain CF1	342×10^4	268.00	33.3×10^6	156.80
GROUNDNUTS	strain ATCC	5440×10^4	trace °	160.0×10^6	0.37
	strain CF1	2640×10^4	89.30	73.3×10^6	22.4
WHEAT	strain ATCC	14×10^3	trace °	11.2×10^6	0.48
	strain CF1	8.2×10^3	0.03	1.2×10^6	2.8
MAIZE	strain ATCC	60.2×10^3	trace °	50.3×10^6	2.4
	strain CF1	12.6×10^3	trace °	440.5×10^6 ***	1.3

* 4.68×10^6 colonies of Penicillium cyclopium.

** 1.57×10^6 colonies of Aspergillus niger.

*** 60.2×10^6 colonies of Aspergillus niger and 120.4×10^6 of Penicillium cyclopium.

° Less than 0.001.

clearly that in each seed there are qualitative differences (Table 2). The sunflower seeds appear the best substrate to produce aflatoxins for both strains assayed. As all the seeds show an evident presence of the fungus externally with a large quantity of conidia and mycelium we also analysed the aflatoxin contents of conidia and mycelium produced on sunflower seeds. From 100 grams of mouldered seeds we obtained with time a higher and higher production of the fungus. Also the production of aflatoxins was evident but did not increase as the growth rate of the fungus (Table 3). It is also evident that the aflatoxin contents in the fungus is irrelevant as compared with the contents in the whole seed. For this reason it appears clearly that the aflatoxins are concentrated fundamentally in the seeds.

The high aflatoxin production on sunflower prompted us to analyse organic fractions which could stimulate or inhibit the growth of the fungus and the aflatoxin production. For this reason we verified the effect of the single fractions after 6 and 12 days both on the fungal growth and the aflatoxin production. For this experiment we tested the strain CF1 that presented a lower growth rate but a higher aflatoxin production on sunflower seeds as compared to the other assayed strain of *A. flavus*. Table 4 shows the microbial growth on the whole seed, on the reconstructed seed including the four fraction, as well as on the reconstructed seed lacking one fraction each time. On the whole seed and on the reconstructed one the growth of *A. flavus* is very similar both at 6 and 12 days of incubation at 30°C. In all the other cases, when the reconstructed seed lacked one fraction we obtained different results. In the absence of carbohydrates fraction and hull the growth was lower compared to the whole seed, whereas in the absence of protein fraction the growth was higher.

Aspergillus flavus was absent in the reconstructed seed lacking the lipid fraction. In this case we only obtained bacteria, which hindered the fungal development. In some cases we also obtained the growth of other fungal strains: *Rhizopus* sp. and *A. niger*. The bacteria were always present.

As shown in Table 5 the production of toxins is not directly connected with the growth of *Aspergillus flavus*. It is clear that the aflatoxin production on the reconstructed seed is much lower than in the whole seed. This fact seems to indicate that the extraction of the organic fractions affects the production of toxins. However every fraction shows a different influence on aflatoxin production. In fact, the hull seems to have an inhibiting effect, whereas protein fraction shows a stimulating effect. The reconstructed seed lacking lipid fraction cannot be considered because the *A. flavus* did not grow at all. The irrelevant amount of toxins found in this case may involve a slight presence of the inoculated fungus, which anyway, was not detected owing to the growth of bacteria.

The detection of other microrganisms (fungi and bacteria) in the *Aspergillus flavus* cultures may make the problem of the aflatoxin production more complicated because of the interfering effect of the presence of the different microrganisms.

TABLE 2 — *Aflatoxins (μg/gram of seed) produced on different moistened seeds inoculated with two strains of Aspergillus flavus after 8 and 21 days of incubation at 30°C.*

SEEDS		AFLATOXINS							
		8th day				21st day			
		B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
ARACHIDS	strain ATCC	0.08	0.006	0.006	—	84.02	10.30	—	—
	strain CF1	0.012	trace*	0.015	0.011	15.40	2.27	61.40	15.25
SUNFLOWER	strain ATCC	0.133	0.006	0.001	—	34.82	3.25	3.53	—
	strain CF1	95.80	3.150	160.05	8.00	37.40	4.28	76.72	38.40
GROUNDNUTS	strain ATCC	trace*	—	—	—	—	—	—	—
	strain CF1	32.06	1.24	32.50	8.50	6.44	—	15.36	0.60
WHEAT	strain ATCC	trace*	—	—	—	0.48	—	—	—
	strain CF1	—	—	0.03	—	—	—	2.8	—
MAIZE	strain ATCC	trace*	—	—	—	2.20	—	—	—
	strain CF1	—	—	trace*	—	—	—	1.30	—

* Trace means less than 0.001.

TABLE 3 — *Aflatoxins produced in the conidia and mycelium of Aspergillus flavus (strain CF1) when the fungus grows on 100 grams of moistened sunflower seeds.*

Days of incubation	Dry weight of conidia and mycelium (mg)	AFLATOXINS (μg) Total	B_1	B_2	G_1	G_2	Aflatoxins/dry weight of conidia and mycelium (μg/mg)
7	382	2.97	0.97	0.05	1.90	0.03	0.0077
14	971	3.20	0.81	0.11	2.23	0.17	0.0032
21	1412	4.22	1.57	0.13	2.06	0.46	0.0029

TABLE 4 — *Growth of different fungi (number of colonies/gram) on the organic fractions extracted from sunflower seeds and on the whole seed after 6 and 12 days at 30°C.*

	DAYS OF INCUBATION 6			12		
	A. flavus	A. niger	Rhizopus sp.	A. flavus	A. niger	Rhizopus sp.
WHOLE SEED	380×10^4	—	2×10^4	2450×10^4	—	11×10^4
CF + LF + PF + H	454×10^4	—	62×10^4	2290×10^4	180×10^4	560×10^4
H + LF + PF	306×10^4	—	—	420×10^4	—	2×10^4
LF + PF + CF	115×10^3	—	—	322×10^4	—	—
H + PF + CF	—	—	3×10^3	—	—	—
LF + CF + H	1038×10^4	24×10^4	—	3010×10^4	12×10^4	2×10^4

Abbr.: PF = Protein Fraction, CF = Carbohydrates Fraction, LF = Lipid Fraction, H = Hull.

* In all cases we have noticed the presence of bacteria.

TABLE 5 — *Aflatoxins production (γg/gr) on the different organic fractions extracted from sunflower seeds and on the whole seed after 6 and 12 days at 30°C.*

	AFLATOXINS									
	6th day					12th day				
	Total	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2
WHOLE SEED	2.67	1.05	0.22	1.12	0.28	34.25	14.01	1.62	16.20	2.42
CF + LF + PF + H	0.33	0.19	trace	0.13	trace	2.44	1.62	0.22	0.60	—
H + LF + PF	0.52	0.23	0.23	0.04	trace	0.40	0.40	—	—	—
LF + PF + CF	0.77	0.66	—	0.09	—	8.00	6.00	trace	1.60	—
H + PF + CF	0.04	0.04	—	—	—	0.35	0.35	—	—	—
LF + CF + H	trace	trace	—	trace	—	—	—	—	—	—

Abbr.: PF = Protein Fraction, CF = Carbohydrate Fraction, LF = Lipid Fraction, H = Hull.

Therefore it is necessary to carry out further experiments concerning the sterilization of whole and reconstructed seeds to verify also the effect of sterilization both on the growth of the fungus and the toxin production.

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