Lipoperoxidation and Aflatoxin Biosynthesis by *Aspergillus parasiticus* and *A. flavus*

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The amount of aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* grown on various aged and non-aged seeds, kept at suitable conditions of temperature and moisture, is particularly related to the peroxide numbers of the seed oils. The addition of synthetic hydroperoxides to the cultures greatly increased aflatoxin production.

INTRODUCTION

Since aflatoxins were discovered, many experiments have been done to study the biochemical and physical factors affecting aflatoxin biosynthesis when toxigenic strains of *Aspergillus flavus* or *Aspergillus parasiticus* grow on different media or substrates (Heathcote & Hibbert, 1978; Maggon *et al.*, 1977). Also, the influence of the degree of aerobiosis, moisture and humidity (Rodricks, 1976), or traces of zinc, molybdenum and other metals (Lee *et al.*, 1966) have been related to the stimulation of aflatoxin biosynthesis. The production of aflatoxins on agricultural crops like rice, wheat, cotton-seeds, maize, coconuts, soybeans and sunflower seeds is well-known (Stoloff, 1977), even if available reports show remarkable differences in the amounts of mycotoxins from the different seeds analysed.

In our previous work we found that aflatoxin production by *A. flavus* is much higher on oil seeds than on starchy seeds (Fabbri *et al.*, 1980) and for this reason we tried to determine whether aflatoxin biosynthesis specifically depended on the most important lipid fractions of oil seeds. Liquid culture media of *A. flavus* were supplemented with synthetic triglycerides, fatty acids and sterols, but these lipid fractions acted as carbon sources (Fanelli *et al.*, 1980) promoting the growth of the fungus without enhancing aflatoxin production (Fanelli *et al.*, 1981).

Very recently, however, we have demonstrated that cerulenin and tetrahydrocerulenin, inhibitors of fatty acid synthetase (D'Agnolo *et al.*, 1973), showed a remarkable effect on aflatoxin production when added to culture medium inoculated with *A. parasiticus*, and it was supposed that the effect might be related to their epoxide structure (Fanelli *et al.*, 1983*a*). This hypothesis was confirmed by our other experiments that showed that lipophilic epoxides (9,10-epoxymethylstearate and 9,10:12,13-diepoxyethylstearate) showed an even greater effect than cerulenin and tetrahydrocerulenin in inducing biosynthesis of aflatoxins (Fanelli *et al.*, 1983*b*).

Lipid epoxides may be formed in limited amounts during the oxidation of unsaturated lipids or during the lipoperoxidation which primarily produces lipoperoxides (lipid peroxide radicals, hydroperoxides) and subsequently many other breakdown products responsible for rancidity in naturally occurring fats (Giuliano & Stein, 1974).

In the present work, synthetic lipoperoxides were added to cultures of *A. parasiticus* and *A. flavus* to see whether they induced aflatoxin production. A range of different seeds, with oils of varying peroxide numbers, were incubated in the presence of different strains of *Aspergillus* and the aflatoxin production was analysed.

METHODS

Fungus culture. *Aspergillus parasiticus* Speare (strain NRRL 2999) was analysed in this study. In some experiments we also used two toxigenic strains of *A. flavus* Link ex Fr. (strains ATCC 22548 and CFI isolated in our Institute from wheat seeds stored in silos). Stock cultures were maintained on Czapek Dox agar (Difco) supplemented with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5 mg l^{-1}) and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (1 mg l^{-1}) at 4°C .

Liperoxidation. Linoleic acid ($\text{C}_{18:2(9,12)}$) (Sigma) was peroxidated with lipoxidase (linoleate: oxygen oxidoreductase EC 1.13.11.12) (Sigma) according to Hamberg & Samuelsson (1965). The peroxidated material was purified by silicic acid chromatography (Hamberg & Samuelsson, 1965).

Agricultural commodities. Seeds of wheat (*Triticum vulgare* var. Manitoba), maize (*Zea mays* var. Decalb 365), peanut (*Arachis hypogaea* var. Bombay), soybean (*Glycine max*) and sunflower (*Helianthus annuus* var. Uniflor 70) of different ages were used. The 'non-aged' seeds were seeds harvested and stored no more than 1–2 months. The different aged seeds were kept in experimental silos in our Institute for up to 3 years without concern for the temperature and humidity of the environment. Fatty acid composition, peroxide number and iodine value were determined according to statutory Italian procedures (Anonymous, 1976).

Culture conditions. Samples containing 10^6 conidia from 15 cultures of *A. flavus* or *A. parasiticus* grown on Czapek Dox medium agar were inoculated on the following.

(a) Synthetic culture medium [Czapek Dox broth plus $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5 mg l^{-1}) and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (1 mg l^{-1})] supplemented with linoleic acid (0.25 mg ml^{-1} or 0.5 mg ml^{-1}) and linoleic acid hydroperoxides (0.25 mg ml^{-1} or 0.50 mg ml^{-1} ; either a non-purified peroxidated linoleic acid mixture or the purified linoleic acid hydroperoxides that consist of about 30% 9-hydroperoxyoctadeca-10,12-dienoic acid and about 70% 13-hydroperoxyoctadeca-9,11-dienoic acid). All the experiments were done in 50 ml medium in 100 ml Erlenmeyer flasks at 30°C .

(b) A 20 g portion of seeds was moistened with enough sterile water to be suitable for the growth of the fungi (Fabbri *et al.*, 1980); as each seed type had its natural moisture, not always optimal for fungal growth, water was added in order to reach the following moisture values: wheat, 18.5%; maize, 21.7%; sunflower, 11%; peanut 11%, soybean, 15%. The seeds were put in 100 ml Erlenmeyer flasks and autoclaved for 20 min at 121°C after which they were inoculated and incubated at 30°C . All the analyses of the seeds were made before autoclaving, which does not significantly affect the levels of lipid peroxides, the iodine value and the fatty acid composition. None of the seeds used in our experiments contained aflatoxins before inoculation.

Estimation of fungal growth. Fungal growth was estimated as previously described (Fanelli *et al.*, 1980).

Analysis of aflatoxins. The aflatoxins were extracted and analysed as described by Fanelli *et al.* (1983a).

Statistics. Data are presented as means $\pm$ S.E.M.

RESULTS

Table 1 reports the fatty acid composition and the properties of oil extracted from non-aged samples of the seeds studied. The iodine values and peroxide numbers were very variable for aged seeds of all types because very different ages were used, but in all cases analysed in our experiments, the iodine value decreased and the peroxide number increased with age. In one sample of 3-year-old sunflower kernels, we found an iodine value of 118 ± 4 and a peroxide number of 17 ± 1.1 . [However, it is not always true that the peroxide number is higher in aged seeds than in non-aged ones; the result may depend on chemical and physical conditions during the storage.]

Table 2 reports aflatoxin production by *A. parasiticus* on non-aged seeds after 15, 30 and 40 d incubation at 30°C . In the aged seeds analysed in our experiments, which had a higher peroxide number than non-aged ones, the aflatoxin production was much higher. The highest production was detected in a sample of aged sunflower seeds showing, after 30 d incubation, $3.2 \pm 0.4 \text{ mg aflatoxins (g seeds)}^{-1}$.

Table 3 reports fungal growth, total lipid content of mycelia and production of aflatoxins by *A. parasiticus* on synthetic media supplemented with different concentrations of linoleic acid ($\text{C}_{18:2(9,12)}$) and peroxidated linoleic acid at 6 and 10 d incubation at 30°C . The presence in the cultures of hydroperoxides greatly increased aflatoxin production. The percentage composition of lipid fractions of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) extracts from mycelia and the fatty acid composition of free fatty acids, triglycerides and polar lipid fractions were also studied. No significant difference was found among all the samples examined (control mycelium and mycelia from cultures supplemented with peroxidated linoleic acid) either in the percentage composition of lipid fractions or in the fatty acid composition of the different lipid fractions.

Table 1. *Fatty acid composition and properties of oils in the non-aged seeds studied*Each value represents the mean $\pm$ S.E.M. of five determinations.

Seed	Percentage of oil extracted from seeds	Fatty acid percentage composition of oil										Iodine value*	Peroxide number†
		C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{20:0}	C _{16:1}	C _{18:1}	C _{18:2}	C _{18:3}	Others		
Soybean	19	0.1 $\pm$ 0.02	0.1 $\pm$ 0.01	10.0 $\pm$ 1.3	2.5 $\pm$ 0.3	0.5 $\pm$ 0.05	0.2 $\pm$ 0.03	24.1 $\pm$ 2.6	53.5 $\pm$ 4.0	8.2 $\pm$ 1.7	0.7 $\pm$ 0.1	133 $\pm$ 4	1.0 $\pm$ 0.03
Peanut	48	—	—	9.1 $\pm$ 0.8	2.3 $\pm$ 0.2	2.2 $\pm$ 0.8	—	54.5 $\pm$ 4.3	29.0 $\pm$ 2.7	—	2.2 $\pm$ 0.4	94 $\pm$ 2	0.8 $\pm$ 0.04
Sunflower	44	—	—	5.5 $\pm$ 0.6	2.6 $\pm$ 0.4	0.2 $\pm$ 0.03	—	16.2 $\pm$ 2.0	75.5 $\pm$ 4.2	—	—	131 $\pm$ 5	1.2 $\pm$ 0.06
Maize	4.2	—	1.2 $\pm$ 0.03	12.9 $\pm$ 0.4	2.9 $\pm$ 0.3	—	0.5 $\pm$ 0.06	49.5 $\pm$ 3.6	33.0 $\pm$ 2.8	—	—	121 $\pm$ 3	0.5 $\pm$ 0.04
Wheat	3.4	0.1 $\pm$ 0.01	0.2 $\pm$ 0.03	15.7 $\pm$ 1.4	2.8 $\pm$ 0.2	0.3 $\pm$ 0.04	—	25.0 $\pm$ 2.6	52.0 $\pm$ 3.1	3.3 $\pm$ 0.5	0.3 $\pm$ 0.04	126 $\pm$ 2	1.0 $\pm$ 0.06

* Expressed as g I₂ per 100 g oil.

† Expressed as milliequivalents active oxygen per 100 g oil.

Table 2. *Aflatoxin production (B₁ + B₂ + G₁ + G₂) by A. parasiticus on non-aged seeds after 15, 30 and 40 d incubation at 30 °C*Each value represents the mean $\pm$ S.E.M. of five determinations.

Seed	Aflatoxin produced [μ g (g seeds) ⁻¹]		
	15 d	30 d	40 d
Soybean	220 $\pm$ 78	380 $\pm$ 130	452 $\pm$ 144
Peanut	132 $\pm$ 45	335 $\pm$ 122	364 $\pm$ 128
Sunflower	350 $\pm$ 53	712 $\pm$ 98	885 $\pm$ 165
Maize	66 $\pm$ 19	113 $\pm$ 21	126 $\pm$ 42
Wheat	75 $\pm$ 10	153 $\pm$ 25	178 $\pm$ 37

Table 3. *Growth, total lipids and aflatoxin production by A. parasiticus grown at 30 °C on synthetic medium supplemented with linoleic acid or peroxidized linoleic acid*

Each value represents the mean $\pm$ s.e.m. of five determinations.

Medium*	Dry weight of mycelium (mg)		Total lipid† (%)		Aflatoxin‡ (µg)	
	6 d	10 d	6 d	10 d	6 d	10 d
SM	309 $\pm$ 27	378 $\pm$ 31	16.3 $\pm$ 3.8	17.5 $\pm$ 3.0	3.3 $\pm$ 0.5	2.4 $\pm$ 0.7
SM + linoleic acid (0.25 mg ml ⁻¹)	351 $\pm$ 57	406 $\pm$ 48	17.9 $\pm$ 4.1	19.2 $\pm$ 2.7	3.9 $\pm$ 0.7	2.7 $\pm$ 0.7
SM + linoleic acid (0.5 mg ml ⁻¹)	391 $\pm$ 40	430 $\pm$ 55	21.7 $\pm$ 3.4	22.6 $\pm$ 4.3	3.6 $\pm$ 0.6	2.9 $\pm$ 0.9
SM + NPPLAM (0.25 mg ml ⁻¹)§	301 $\pm$ 35	408 $\pm$ 37	18.3 $\pm$ 3.6	18.9 $\pm$ 3.0	137.5 $\pm$ 38.4	322.8 $\pm$ 75.8
SM + NPPLAM (0.5 mg ml ⁻¹)§	325 $\pm$ 51	418 $\pm$ 43	19.6 $\pm$ 3.8	21.2 $\pm$ 3.8	150.4 $\pm$ 53.6	807.2 $\pm$ 103.5
SM + linoleic acid hydroperoxides (0.25 mg ml ⁻¹)	272 $\pm$ 41	395 $\pm$ 41	18.8 $\pm$ 3.2	19.2 $\pm$ 2.8	102.3 $\pm$ 49.5	417.5 $\pm$ 94.6
SM + linoleic acid hydroperoxides (0.5 mg ml ⁻¹)	258 $\pm$ 37	407 $\pm$ 53	19.1 $\pm$ 3.6	19.6 $\pm$ 3.9	90.5 $\pm$ 41.7	899.3 $\pm$ 127.4

* SM, synthetic medium; NPPLAM, non-purified peroxidized linoleic acid mixture.

† Total lipid is expressed as mg total lipids extracted with CHCl₃/CH₃OH (2:1, v/v) from mycelium per mg dry weight of mycelium $\times$ 100.

‡ Aflatoxins = total aflatoxins (B₁ + B₂ + G₁ + G₂); the most important quantitatively were in all cases B₁ and G₁.

§ In this experiment the NPPLAM contained about 30% linoleic acid. The unchanged linoleic acid was analysed according to Passi *et al.* (1981).

|| According to Hamberg & Samuelsson (1965), linoleic acid hydroperoxides consist of about 30% 9-hydroperoxyoctadeca-10,12-dienoic acid and about 70% 13-hydroperoxyoctadeca-9,11-dienoic acid. They were not further investigated.

This confirmed our previous work (Fanelli *et al.*, 1983*a, b*). The experiments carried out with the toxigenic strains of *A. flavus* (ATCC 22548 and CF1) confirmed the stimulation of aflatoxin biosynthesis by hydroperoxides. For example, the two strains of *A. flavus* after 10 d incubation at 30 °C on synthetic medium (50 ml) supplemented with non-purified peroxidized linoleic acid mixture (NPPLAM) (0.5 mg ml⁻¹), produced total aflatoxins as follows: strain ATCC 22548, 651.0 ± 151.3 µg; strain CF1, 778 ± 188.2 µg. In all cases, the quantitatively most important aflatoxins were B₁ and G₁.

DISCUSSION

From our results it appears that the oxidation of unsaturated lipids plays a fundamental role in the induction of aflatoxin production by *A. flavus* and *A. parasiticus*. In previous work (Fanelli *et al.*, 1983*b*) we also demonstrated that synthetic epoxides, at about the same concentration as lipoperoxides, induced aflatoxin production in cultures of *A. flavus* and *A. parasiticus*. But, whereas epoxides may form to a limited extent during the oxidation of unsaturated lipids, both *in vivo* and *in vitro*, the main products are lipoperoxides which can undergo further degradation. It is well known that the tendency of unsaturated lipids to absorb oxygen is directly dependent on the degree of unsaturation. In fact, while oxygen uptake is very low in mono-unsaturated lipids, polyunsaturated lipids bind oxygen even at room temperature in daylight. The problem is further complicated by the fact that many other chemical and physical factors in the environment may act on lipoperoxidation: radiation, temperature, humidity and the presence of various metal salts and antioxidants.

The amount of aflatoxins produced by *A. parasiticus* on various non-aged seeds, kept under suitable conditions of temperature and moisture, was higher on oily seeds than on starchy seeds (Table 2). In addition, the percentage of polyunsaturated fatty acids (linoleic and linolenic acid) in the oils was important, probably because these fatty acids are more easily peroxidizable than the mono-unsaturated fatty acids. In fact, sunflower seeds (75.5% of linoleic acid in their oil) and soybean (53.5% of linoleic acid and 8.2% of linolenic acid in their oil) permitted the production of a higher amount of mycotoxins than peanut (29% of linoleic acid). Also in the starchy seeds, aflatoxin production was higher on wheat, which has a greater percentage of polyunsaturated fatty acids (52.3% of linoleic acid and 3.3% of linolenic acid) than on maize (33% of linoleic acid). The induction of aflatoxin production by lipoperoxides might explain the differing results of many authors on aflatoxin contents in various agricultural products.

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