EFFECT OF DIFFERENT BY-PRODUCTS OF LIPOPEROXIDATION ON THE AFLATOXIN BIOSYNTHESIS

C.FANELLI[1] , A.A.FABBRI[1], R. CASTORIA[1], G. PANFILI[2] and C. DE LUCA[3]

1, Dipartimento di Biologia vegetale, Università di Roma 'La Sapienza',Largo Cristina di Svezia 24, 00165 Roma, Italy.

2, Arba 87, Vicolo Orti della Magliana 29, 00148 Roma, Italy.

3, Istituto San Gallicano 25a, 00154 Roma, Italy.

ABSTRACT

Lipoperoxides(epoxides, hydroperoxides etc.) from any sources are capable of over stimulating the production of aflatoxins and their congeners both 'in vitro' and 'in vivo'. It is well known that hydroperoxides from polyunsaturated fatty acids are very unstable compounds and decompose by chain cleavage to form complex mixtures of compounds such as aldehydes, ketons, alkanes and short chain carboxylic acids. Some of these by-products, namely hexanal, pentanal, 2-nonenal, 2-octenal, caproic acid(from 0.01% to 0.05%)were found to parallel the effect of lipoperoxides in supporting the production of aflatoxins 'in vitro'. Acetic, propionic and iso-butyric acid up to 0.1% show a clear stimulating effect on the aflatoxin production. The effect of propionic acid added to starchy and oily seeds is fungistatic from 0.2% in wheat and from 0.3% in sunflower seeds.

INTRODUCTION

In the recent years a wide number of antifungal compounds have been studied to prevent and limit mould growth and mycotoxin production in different high moisture grains and oily seeds. For this purpose propionic acid has been suggested by a number of investigators (Christensen, 1974; Vandergraft et al.1975), but recommendations differ widely regarding the concentration necessary for the effective fungal control(Tsai et al. 1984, Stewart et al., 1977, Ghosh et al., 1982; Ghosh and Haggblom, 1985; Zaika and Buchanam, 1987). In addition Al-Hilli and Smith(1979) found no inhibition of Aspergillus flavus growth in propionic acid at 500-1000 mg/l after 9 days 'in vitro', and in addition demonstrated 'in vitro' stimulation of aflatoxins production by sublethal concentrations of propionic acid.

It is also important to considere that hydroperoxides from any sources are capable of over-stimulating aflatoxin production both 'in vitro' and 'in vivo', and are very unstable compounds and

decompose by chain cleavage to form complex mixtures of aldehydes, ketones, alkanes and carboxylic acids etc. some of which have been implicated in diseases such as cancer (Esterbauer, 1982).

Sometimes toxigenic Aspergilli have been important colonizers of grain treated inadequately with propionic acid and have formed aflatoxins (Hackins & Biggs, 1979), perhaps stimulated by the propionic acid (Al -Hilli & Smith, 1979) or by other microrganisms colonizing the grain (Cuero et al. , 1987a,b).

The aim of this work was to evaluate whether by-products of lipoperoxidation (BPLP= aldehydes, ketones, short chain carboxylic acids) behave like lipoperoxides in supporting aflatoxin production. In addition we have tested different sublethal concentrations of propionic acid added to wheat and sunflower seeds inoculated with an aflatoxigenic strain of A.parasiticus . Fungal growth and aflatoxin production were followed during the experiments.

MATERIALS AND METHODS

Organism and culture conditions

A toxigenic strain of Aspergillus parasiticus(strain SRRC 2004), a generous gift from dr.Maren A.Klich from U.S.Department of Agriculture and prof. Joan Bennett, Tulane University, New Orleans was used. The fungus produced aflatoxins B_1, B_2, G_1 and G_2. Stock culture were grown at 30°C on modified Czapek Dox agar medium (Czapek Dox agar + 5 mg/l $ZnSO_4 \cdot 7 H_2O$ and 1 mg/l $Na_2MoO_4 \cdot 2 H_2O$). About 10^6 conidia from 15-day-old cultures were incubated in 50 ml of the above-modified synthetic medium.

In the first series of experiments after 5 days at 30°C the cultures were supplemented at concentrations ranging from 0.01 to 0.05% w/v with pentanal, hexanal, 2-nonenal, 2-octenal, 2-heptanal, caproic acid and 2-butanone, and at concentrations ranging from 0.01 to 0.1% with acetic, propionic, butyric, isobutyric and valerianic acids.

In a second series of experiments wheat(cv.kreso) and sunflower(cv.Maxflor) seeds were moistened up to 20% by adding sterile water. After equilibration overnight 100 ml Erlenmeyer flasks with 10g of the non-sterilized seeds were seeded with $4x10^6$ conidia from 15-day-old cultures on Czapek Dox agar of A.parasiticus and with different concentrations of propionic acid (from 0.05 to 0.6%).

Analytical techniques

The fungal growth in 'in vitro' experiments (dry weights) and the total aflatoxin production were determined as previously described (Fanelli et al., 1983).

The fungal growth on wheat and sunflower seeds was measured using an arbitrary scale: 0: no visible mould, 1: isolate mouldy grains, 2: up to half grains colonized, 3: heavy molding. The aflatoxin production was determined as previously described (Passi et al., 1987).

RESULTS

The Table I clearly shows that all the assayed by-products of lipoperoxidation (BPLP) (i.e. hexanal, pentanal, 2-nonenal, 2-octenal, 2-heptenal, 2-butanone and caproic acid) at concentration ranging from 0.01% w/v to 0.05 w/v do not affect mycelial growth but show an evident effect on aflatoxin production. The stimulation is dose dependent up to 0.05% w/v concentration of active substances, higher amounts can lead to the death of fungus and therefore were not reported.

Table I. Effect of different concentrations (0.01% w/v and 0.05% w/v) of some by-products of lipoperoxidation on fungal growth and aflatoxin production by A.parasiticus(SRRC 2004) grown on synthetic medium at 30°C for 15 days. Each result represents the mean ± SD of three determinations.

Compounds	Concentration (%)	dry weight (mg/50 ml)	total aflatoxins (μg/50 ml)
None	-	260 ± 21	4.5 ± 0.5
Hexanal	0.01	270 ± 32	112.4 ± 22.9
	0.05	290 ± 38	186.5 ± 29.7
Pentanal	0.01	255 ± 28	107.5 ± 19.9
	0.05	233 ± 19	170.7 ± 18.7
2-Heptenal	0.01	240 ± 38	142.7 ± 28.6
	0.05	222 ± 18	326.6 ± 42.5
2-Octenal	0.01	277 ± 32	128.1 ± 19.3
	0.03	244 ± 22	307.8 ± 33.5
Caproic acid	0.01	255 ± 21	34.4 ± 4.5
	0.05	288 ± 30	84.5 ± 11.1
2-Butanone	0.01	252 ± 22	90.6 ± 8.6
	0.05	240 ± 18	135.6 ± 20.5

In the Fig.1 are reported the effect of different short chain carboxylic acids : acetic (C_2), propionic (C_3), butyric (C_4), iso-butyric (C_{4iso}), valerianic(C_5) and caproic acid(C_6) at 0.05% and 0.1% w/v (when the higher concentration was no toxic). In the figure there are reported also the data concerning the presence of HCl (1.5 and 3.0 mM) in the culture to evaluete the effect of pH on the production of aflatoxins, considering that the addition of the carboxilic acids increase the acidity in the medium.

As shown for many by-products of lipoperoxidation and organic acids occurring in food as a result of natural processing (Buechat and Golden,

1989). the concentration of 0.1% is toxic for the fungal growth for butyric, valerianic and caproic acid. On the contrary, acetic, propionic and iso-butyric acid at 0.1% show a clear stimulating effect on the aflatoxin production.

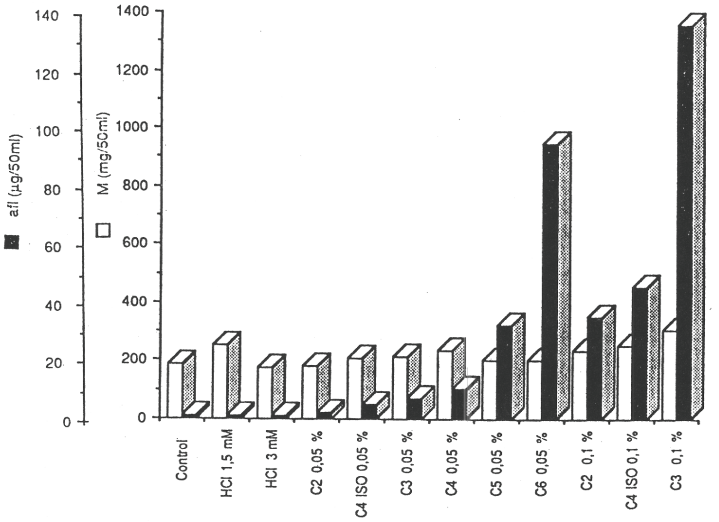

Fig.1. Effect of different short chain carboxylic acids on aflatoxin production and fungal growth.
afl = total aflatoxins; M= mycelium dry weight

Tables 2 and 3 show the effect of different concentrations of propionic acid from 0.05% to 0.6% both on fungal growth and aflatoxin production in seeds inoculated with A.parasiticus . It appears clearly that on wheat seeds the concentration of propionic acid show an evident inhibiting effect both on fungal growth and aflatoxin production. In sunflower seeds the complete inhibiting effect was obtained with 0.3% of propionic acid.

DISCUSSION AND CONCLUSIONS

Among the BPLP the saturated aldehyde, hexanal, can be considered the most important molecule, in fact it is the major carbonylic compound from the peroxidated linoleic, γ-linolenic and arachidonic acid (Esterbauer, 1982, Logani and Davis, 1980). It is noteworty that, among the aldehydes, alkenals are more effective than alkanals in inducing the output of mycotoxins probably because of their biological reactivity, for example, against those biomolecules which represent the antioxidant pool of the cells such as cysteine, reduced glutathione, proteins and enzymes containing -SH groups (Esterbauer, 1982).

Furthermore, hexanal, which is biosynthetized in plant by the action of lipoxygenase and hydroperoxide lyase on linoleic acid, inhibited the germination and subsequent growth of soybean (Garner et al., 1990).

Table II- Effect of different concentrations of propionic acid(from 0.05 to 0.6%) on fungal growth and aflatoxin production on wheat seeds (moistened to 20%) inoculated with A.parasiticus(SRRC 2004) and incubated at 30°C for 7 and 14 days. Each result represents the mean ±SD of five determinations.

Propionic ac.concentration (%)	7 days fungal growth	7 days aflatoxins (μg/10g)	14 days fungal growth	14 days aflatoxins (μg/10g)
control	3	15.2±2.2	3	48.2±5.5
0.05	3	16.4±2.4	3	46.2±4.9
0.1	3	22.4±3.3	3	55.8±6.6
0.2	0	-	0	-
0.3	0	-	0	-
0.4	0	-	0	-
0.6	0	-	0	-

Table III-Effect of different concentrations of propionic acid(from 0.05 to 0.6) on fungal growth and aflatoxin production on sunflower seeds (moistened to 20%) inoculated with A.parasiticus(SRRC 2004) and incubated at 30°C for 7 and 14 days). Each result represents the mean ± SD of five determinations.

Propionic ac.concentration (%)	7 days fungal growth	7 days aflatoxins (μg/10g)	14 days fungal growth	14 days aflatoxins (μg/10g)
control	3	35.5±4.5	3	66.6±7.4
0.05	3	38.0±4.7	3	69.4±6.9
0.1	3	48.5±5.0	3	80.8±9.3
0.2	3	58.2±4.4	3	90.2±8.6
0.3	0	-	0	-
0.4	0	-	0	-
0.6	0	-	0	-

A lower reactivity of caproic acid and 2-butanone in comparison with aldehydes may explain the lowering of aflatoxin production.

In the experiments with starchy and oily seeds inoculated with A.parasiticus we have found that wheat seeds are more affected by propionic acid both as fungal growth and aflatoxin production.

It must be noted that the effect of propionic acid is fungistatic from 0.2% in wheat and from 0.3% in sunflower seeds(both seeds moistened to 20%) inoculated with A.parasiticus. If we used higher concentrations no fungal growth aflatoxin was supported for two weeks, indicating that such doses are more effective in the inhibition of fungal growth. Lower concentrations of propionic acid after a lag phase of some days induce a fungal growth and a slight increase of aflatoxin production than the seeds without the drug.

However conflicting reports concerning the effective concentrations of propionic acid inhibiting the A.flavus and A.parasiticus, the aflatoxin production and its metabolism in the cell made us interested to clarify the molecular mechanism of action of propionic acid.

The mechanism of inhibition of most antimicrobial food additives(such as lipophilic acids) prevent growth by inhibiting transport of substrate membrane into the cells.

In our opinion propionic acid can be metabolized by the fungus and may represent the starter of odd monocarboxylic fatty acids.

In conclusion: chain length, degree of unsaturation , geometric configuration and molecular reactivity are all important determinants of BPLP toxicity.

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EFFETS DES DIFFERENTS SOUS-PRODUITS DE LA LIPOPEROXYDATION SUR LA BIOSYNTHESE DE L'AFLATOXINE

C. FANELLI, A.A. FABBRI, R. CASTORIA, G. PANFILI et C. DE LUCA

Dipartimento di Biologia Vegetale, Univ. Roma "La Sapienza"
Largo Cristina di Svezia 24, 00165 Roma, Italia

RESUME

Les lipopéroxydes (époxydes, hydropéroxydes) de toutes provenances sont capables de sur-stimuler la production d'aflatoxines et des substances apparentées, à la fois *in vitro* et *in vivo*. Il est bien connu que ces hydropéroxydes des acides gras polyinsaturés, une étape cruciale dans le processus lipopéroxydatif, sont des substances très instables qui se décomposent par clivage de leurs chaînes pour former des mélanges complexes de composés tels qu'aldéhydes, cétones, alkyles et acides carboxyliques. Quelques uns de ces sous-produits, nommés hexanal, pentanal, 2-nonénal, 2-octénal (à des concentrations entre 0,05 % et 0,01 % w/v) et acide propionique, acide acétique, acide butyrique, acide valérique et acide caproïque (à des concentrations entre 0,05 % et 0,01 % v/v) ont des effets parallèles aux effets des lipopéroxydases qui interviennent dans la production des aflatoxines.

A la suite de ces résultats, nous avons examiné les effets de l'acide propionique (à différentes concentrations entre 0,1 % et 0,3 % v/v) seul ou en combinaison avec 0,5-4,0 KGy d'irradiation sur froment et graines de tournesol correctement humidifiées et après inoculation de 10^6 de conidies d'une souche toxinogène d'*Aspergillus parasiticus* (NRRL 2999). Après 14 jours d'incubation, la croissance fongique et la production d'aflatoxines se sont avérées évidentes, jusqu'à une concentration de 0,2 % d'acide propionique dans les deux semences et une concentration plus élevée pour le tournesol que pour le blé. En augmentant les concentrations d'acide propionique (0,3 % pour le froment et 0,4 % pour le tournesol), l'effet inhibiteur sur la croissance fongique et sur la production d'aflatoxines est alors devenu évident.