Mould Growth and Aflatoxin Accumulation Affected by Newly Synthesized Derivative of Coumarine Treatment of Maize Hybrid

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Summary

The effect of newly synthesized derivative of 11-hydroxy-7-imino-7H-7a, 12-diazabenzo/α/anthracen-6-one, on growth and aflatoxin B_1 and G_1 (AFB_1 and AFG_1) accumulation by toxigenic mould *Aspergillus flavus* ATCC 26949 was studied on a solid substrate (maize grains) to determine the possible use of this compound as a mean of controlling aflatoxin accumulation. Experiments were carried out in a stationary culture at temperature of 28°C during 21 days. The growth of mould was monitored by measuring the analysis of chitin as glucosamine, as a criterion, and concentration of AFB_1 and AFG_1 was measured by HPLC method using Hewlett-Packard instrument with flourescence detector. Concentration of investigated coumarine of 0.05 mmol x g^{-1} stimulated mould growth and aflatoxin accumulation, but concentration of 0.2 mmol x g^{-1} or higher produced an inhibitory effect. In the presence of 0.2 and 0.5 mmol x g^{-1} of this compound, mould growth was decreased by 22% and 65%, respectively. Concentration of AFB_1 in these investigations was reduced by 30% and 90%, and concentration of AFG_1 was reduced almost completely in respect to values obtained in control experiments. In experiments with 2.0 mmol x g^{-1} of this inhibitor no synthesis of both investigated aflatoxins was found in the sample although small increment of biomass was detected.

Key words

moulds, chitin, aflatoxins, antifungal agents, coumarine

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Received: October 29, 2010 | Accepted: December 5, 2010

ACKNOWLEDGEMENTS
The authors would like to thank Professor E.B. Lillehoy, United States Department of Agriculture, Peoria, Illinois for supplying the strain of *Aspergillus flavus* used in this work. We also grateful to Professor M. Terzić for analyses of aflatoxins in maize grains and for information, and Professor V. Rapić for providing of investigated coumarine. Finally, the authors gratefully acknowledge gentlewoman Brigitta Duraković for language correcting the manuscript. The investigations were supported by a grant No. 058-0582184-0432 from Croatian Ministry of Science and Technology.

Introduction

Foods and feeds are frequently contaminated with moulds during harvesting, storage and handling before reaching the consumer.

Many of these ubiquitous moulds have been shown to produce toxins. The presence of known toxin-producing moulds does not necessarily indicate the presence of mycotoxins, nor does the lack of a mould indicate that the sample has not been contaminated with a mycotoxin. Some moulds produce toxins under restricted conditions and only particular strains of some species produce toxins (Pittet, 1998).

Among the fungal toxins associated with human and animal food materials are included several derivatives of coumarin that avoke varied pharmacological and physiological responses in the animal body. Aflatoxins are toxic, highly carcinogenic secondary metabolites produced by the fungal genera *Aspergillus*, *Penicillium* and *Rhizopus* (Goldblatt, 1969; Brown *et al*., 1999; Dorner *et al*., 1999; Wu, 2004; Binder, 2007). AFB_1 has been associated with certain mycotoxicoses including turkey "X" disease and chronic exposure to low levels of this toxin pose a serious health and economic hazard (Diener *et al.,* 1987; Karlowsky, 1999; Thompson and Henke, 2000; CAST Report, 2003).

To prevent aflatoxin formation in agricultural commodities it is first necessary to prevent the growth aflatoxin producing fungi (Windham *et al.,* 1999; Wright *et al.,* 2000; Schatzmayr *et al*., 2006). This can be achieved by at least three means: control of the environment, use of chemical antifungal agents and utilization of natural resistance in agricultural commodities (Duraković *et al*., 1985; Sušac *et al.,* 1989; Samarajeva *et al*., 1990; Praxton, 1991; Gourama and Bullerman, 1995; Cho and Kang, 2000; Wright *et al.,* 2000; Delaš *et al.,* 2008a, 2008b, 2008c; Duraković *et al.,* 2008). During the past 40 years there has been increasing interest in the identification of naturally occurring or newly synthesized chemical compounds that inhibit growth and/or aflatoxin production by aflatoxigenic species (Hitokoto *et al*., 1980; Duraković *et al*., 1985; Sušac *et al.,* 1989; Duraković *et al.,* 2006; Duraković, 2007; Duraković *et al.,* 2007; Duraković *et al.,* 2008; Duraković *et al.,* 2010).

Preliminary studies in Laboratory of Microbiology, Faculty of Food Technology and Biotechnology, Zagreb, had indicated the efficacy of such derivatives of coumarine as the inhibitors of the growth aflatoxin-producing fungi (Sušac *et al.,* 1989; Duraković *et al.,* 2006; Delaš *et al.,* 2008a, 2008b, 2008c). This was the basis of our study, which aimed at evaluating the mould growth and aflatoxin accumulation during the growth of *A. flavus* ATCC 26949 on maize in the presence of newly synthesized derivative of coumarine (Duraković, 2007; Duraković *et al.,* 2007; Delaš *et al.,* 2008a).

Materials and methods

Culture and inoculum. *Aspergillus flavus* ATCC 26949 strain was obtained from the USDA Fermentation Laboratory Northern Regional Research Center, Peoria, IL. The cultures were maintained on slants of potato dextrose agar (PDA) stored at 4°C. Before each experiment, the mould was transferred to another PDA slant and was incubated at ambient temperature.

The inoculum for all experiments was prepared by growing *A. flavus* on PDA in 100 x 15 mm Petri dishes for seven days at 28°C until it was well sporulated. Contents of two Petri dishes, including agar and fungus, were homogenized in 50 mL sterile water with a Poter homogenizer for 1-2 minutes. The culture was shaken vigorously for 1-2 minutes, and spore number was determined using a Thoma counting chamber. The spore suspension was diluted to 5 x 10^8 conidia x mL^{-1}.

Assay of antifungal activity. The antifungal activity of investigated coumarine was assayed using two techniques as follows:

a) **Standard assay**: 1 mL of the spore suspension of *A. flavus* ATCC 26949 was used to seed the Petri dishes, prepared by pouring 20 mL of PDA. Agar medium was poured into dishes, and the content of the dish mixed well. After allowing the agar to solidify, dishes were prepared using a sterile glass tube and pipette bulb. The various concentrations of investigated coumarine were then added to the dishes in appropriate quantities in triplicate. In each case, a separate dish with pure solvent was employed as a control. The diameter of zone inhibition was measured after incubation of 48 hours at temperature of 25°C.

b) **Assay in flask cultures**: The studies of aflatoxin accumulation by *A. flavus* were carried out in duplicate in Erlenmeyer flasks contained 50 g of maize grains. The water content in the substrate was adjusted by adding appropriate amounts of distilled water to the samples to quantity of 40%. Substrates were autoclaved and then inoculated with 1 mL of the spore suspension containing 5 x 10^8 conidia x mL^{-1} of *A. flavus*. On the basis of preliminary information obtained from the dishes assay, appropriate quantities of various concentrations of coumarine were added in triplicate to the flasks containing maize grains and spores. The flasks were kept for 24 hours with thorough agitation so that the coumarine could be absorbed by grains. Flasks not containing coumarine served as a control. Flasks were incubated at 25°C for 15 days. A method of measuring the degree of fungal invasion on corn was analysis of chitin, measured as glucosamine (Donald and Mirocha, 1977).

Substrate for mould growth and aflatoxin accumulation. The basal substrate used in this study was whole maize grains (ZG-SK 502A hybrid). Fifty grams of grains were distributed in each 300 mL Erlenmeyer flasks. Sterile distilled water (about 22 mL x 50 g-1) was added to achieve moisture content of 40%. The investigated coumarine was synthesized in the Laboratory of Organic Chemistry of Faculty of Food Technology and Biotechnology in Zagreb, and was dissolved in dimethylsulfoxide (DMSO) + water (1 : 1 w/w) at 1.0 mol x L-1 concentration. Its structure is shown in Figure 1.

A method for the synthesis of this compound is described by Govori *et al*., 2002 and Chen *et al.,* 2010. The required amounts of this solution to give 0.05; 0.2; 0.5; 1.0 and 2.0 mmol x g^{-1} of broth were pipetted into test Erlenmeyer flasks. Control test flasks and duplicate test flasks containing the various concentrations of coumarine were inoculated with 1.0 mL of the spore suspension. All the flasks were incubated at 28°C for 21 days.

Measurement of fungal growth. Every seven days control flask and duplicate test flasks were taken out of the incubator as

samples for the determination of the amount of biomass and the AFB1 and AFG1 accumulation. The substrates were autoclaved at 121oC for 30 minutes before analysis to kill the spores and vegetative mycelia. The growth of A. flavus was monitored by using the analysis of chitin measured as glucosamine, as a criterion (Donald and Mirocha, 1977). Chitin, a polymer of N-acetyl-D-glucosamine, is a constituent of the cell walls of most fungi and can be used as a measure of total fungal growth, since little or no chitin-like materials occur in sound cereal grains. In the analytical devises, the polymer is not measured directly but rather is hydrolysed to glucosamine, deaminated to its corresponding aldehyde and measured spectrophotometrically. The chitin content is estimated from the standard curve of glucosamine-HCl read at 650 nm.

On the basis of the data obtained, the calibration curve was made, from which was, according to the chitin content, directly determined amount of biomass dry weight (Duraković, 1981).

The percentage inhibition of mould growth was determined according to the formula:

$$\% \text{ inhibition} = (1 - \frac{m_1}{m_2}) \times 100$$

m_1 = biomass dry weight in flask containing inhibitor (mg x g^{-1})
m_2 = biomass dry weight in control flask (mg x g^{-1})

Aflatoxin assay. The aflatoxins were extracted from samples with chloroform as described by AOAC, 2005. The chloroform was evaporated under nitrogen gas, the samples were dissolved in methanol and filtered through 0.25 µm organic solvent filter (Millipore Co., Bedford, Mass., USA) prior High Performance Liquid Chromatography (HPLC).

To confirm the identity of aflatoxins Thin Layer Chromatography (TLC) was used. Silica gel precoated plates (250 µm thick; Merck, Darmstadt, Germany) were activated at 115oC for one hour before use. TLC plates were developed in chloroform : acetone : petroleum ether (33 : 6 : 1) (modified Duraković *et al.*, 1985), where after they were examined by comparing the R_f values of a known standards to the unknown samples under UV light at 365 nm. Aflatoxin primary standards to check the linearity were provided by Carl Roth (Karlsruhe, Germany). Six milliliter vial of methanol solution with the following specification: 250 ng x mL^{-1} of AFB_1 and 250 ng x mL^{-1} of AFG_1. Working standard solutions of 0.25 and 0.125 ng x mL^{-1} of each aflatoxin (AFB_1 and AFG_1) were prepared by diluting the primary standard solution with methanol-acetonitrile (1 : 1 v/v).

HPLC analysis was performed by using a Hewlett-Packard 1050 Liquid Chromatography (pump and injection system), (Walborn, Germany) with JASCO FP-920 fluorescence detector (Co. Ltd., Japan) and HP integrator 3395. The HPLC column was C_{18} Nova-Pak (4.6 x 250 mm) with particle size of 5 µm (Waters, Millipore; Millford, MA). Detection of aflatoxins was carried out at $lambda_{ex}$ 360 nm and $lambda_{em}$ 420 nm. For aflatoxins the fluorescence detector was set at 360 and 420 nm and the mobile phase consisted of a mixture of deionised water-acetonitrile-methanol (60 : 25 : 15 v/v). The flow rate was 1 mL x min^{-1} for each mobile phase and the injected volume of working standards was 50 µL.

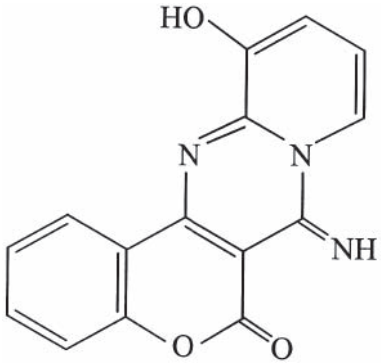

Figure 1. Structure of investigated coumarine (Sušac *et al.*, 1989; Govori *et al.*, 2002; Duraković *et al.*, 2006; Duraković, 2007).

The change of aflatoxin mass fraction (%) is determined according to the formula:

$$\text{concentration change} = (1 - \frac{Y_1}{Y_2}) \times 100$$

Y_1 = mass fraction of aflatoxin in substrate containing inhibitor (µg x g^{-1})
Y_2 = mass fraction of aflatoxin in control substrate (µg x g^{-1})

Results and discussion

The influence of investigated coumarine on the growth of the mould *A. flavus* ATCC 26949 and aflatoxin accumulation are indicated in Table 1 and Figure 2.

Mould growth and AFB_1 and AFG_1 accumulation were observed for 21 days at temperature of 28oC after inoculation of *A. flavus* on whole maize grains. According to previous findings (Gourama and Bullerman, 1995; Wicklow *et al.*, 1998; Weinderbörner, 2001) the optimal temperature for the biosynthesis of AFB_1 is 28-32oC, whereas for AFG_1 it is 24-28oC.

The increased interest in biopreservation of food system has recently led to the development of new synthesized antimicrobial compounds having different origins. A variety of chemical compounds to prevent spoilage by moulds and biosynthesis of mycotoxins have been investigated. These include herbs, spices, essential oils, pesticides, microbial metabolites (bacteriocins and organic acids) and/or by adding fungal inhibitors such as propionic acid, newly synthesized derivatives of dehydroacetic acid, tetraketone and coumarine (Sušac *et al.*, 1989; Samarajeva *et al.*, 1990; Wilkins and Board, 1991; Duraković *et al.*, 1994; Duraković *et al.*, 1995; Basappa and Shantha, 1996; Georgopapadakou, 1998; Duraković *et al.*, 1999; Cho and Kang, 2000; Lavermicocca *et al.*, 2003; CODEX: CAC/RCP 51-2003, 2005; Duraković *et al.*, 2004; Duraković *et al.*, 2006; Duraković, 2007; Delaš *et al.*, 2008a; Parameshwarappa *et al.*, 2009).

Thus Bullerman *et al.* (1977) investigated the antifungal properties of cinnamon in media and raisin bread. They reported the spice to inhibit the growth of *Aspergillus parasiticus* and aflatoxin production; toxin production being inhibited to a greater extent than mycelial growth. Research by Sušac *et al.*

Table 1. Effect of investigated coumarine on growth and aflatoxin accumulation by *Aspergillus flavus* ATCC 26949

Coumarine (mmol x g^{-1})	Incubation time (days)	Biomass dry weight (mg x g^{-1})	Aflatoxin (μg x g^{-1})		%Inhibition[a100]		
			B_1	G_1	Growth	Aflatoxin	
						B_1	G_1
0	7	27.80	640.0	65.0	–	–	–
	14	30.0	1560	105.0	–	–	–
	21	47.50	1000	185.0	–	–	–
0.05	7	28.30	720.0	80.0	Mould growth and accumulation of aflatoxins were stimulated		
	14	34.0	1850	130.0			
	21	52.0	1050	190.0			
1.0	7	9.60	c_	c_	65.0	c_	c_
	14	12.40	170.0	c_	60.0	90.0	c_
	21	14.20	65.0	c_	70.0	95.0	c_
2.0	7	b_	c_	c_	c_	c_	c_
	14	b_	c_	c_	c_	c_	c_
	21	5.70	c_	c_	99.88	c_	c_

a_{100} – (test culture/control culture x 100); b_ - No detectable growth: c_ - No detectable aflatoxin

(1989) and Duraković *et al.* (2006., 2007) on *A. parasiticus* NRRL 2999 and *A. flavus* ATCC 26949 in maize shows that some new synthesized derivatives of coumarine possess an effective antifungal and antimycotoxigenic properties. Our previous results demonstrate clearly that mould growth and aflatoxin accumulation were partially stimulated by amounts of investigated tetraketone and coumarine derivatives near the minimal inhibitory concentrations (Duraković *et al.,* 1995, 2006).

Table 1 and Figure 2 show the results of growth of mould *Aspergillus flavus* ATCC 26949 on maize grains at incubation temperature of 28°C in the presence of different concentrations of investigated coumarine. Duraković *et al.* (2006) investigated the antifungal and antiaflatoxigenic properties of some newly synthesized derivatives of coumarine during the growth of mould *Aspergillus parasiticus* NRRL 2999 in YES medium.

Results obtained in our study confirmed that one of selected and investigated coumarines is an effective inhibitor of synthesis of biomass and accumulation of AFB_1 and AFG_1 during the growth of mould *Aspergillus flavus* ATCC 26949 on maize grains.

In control experiments the greatest amount of biomass obtained after 21 days during the growth of mould culture was 47.50 mg x g^{-1} of substrate. The concentration of AFB_1 reached peak values after 14 days and AFG_1 after 21 days. Maximum concentrations of investigated toxins amounted to: 1560 μg AFB_1 x g^{-1} mycelium dry weight, and 185.0 μg AFG_1 x g^{-1} mycelium dry weight (Table 1).

The effect of increasing concentration of investigated inhibitor on the growth of mould *A. flavus* and aflatoxin accumulation were evaluated. Concentration of coumarine of 0.05 μg x g^{-1} of maize grains produced no inhibitory effect, even stimu-

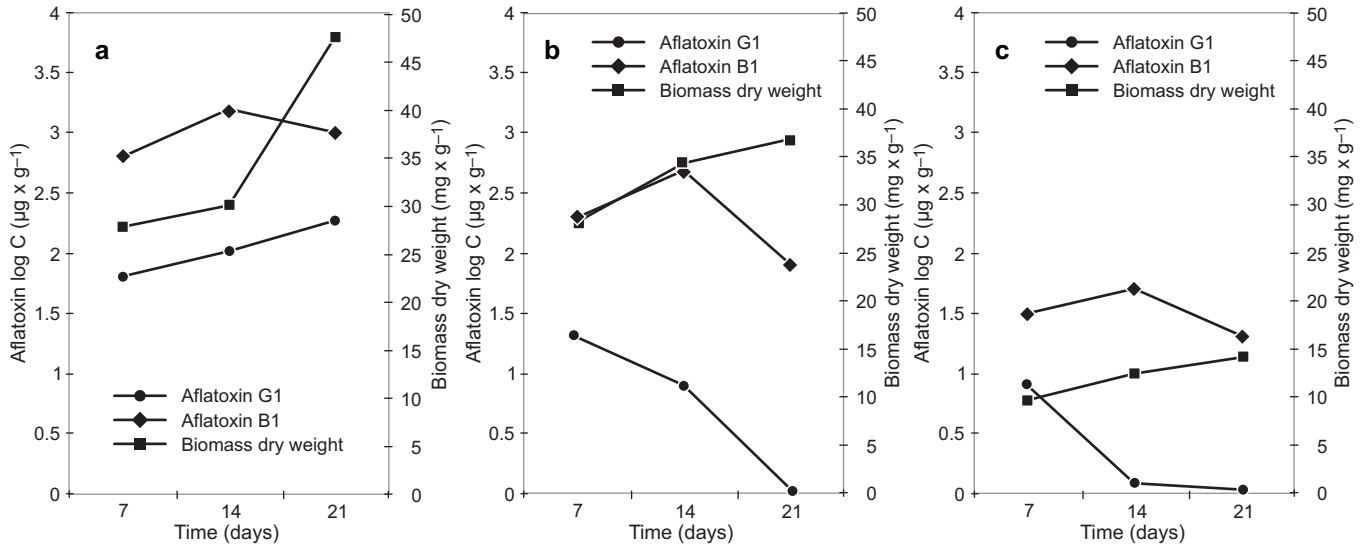

Figure 2. (a, b and c) Comparative presentation of biomass and synthesis of aflatoxins during the growth of mould *Aspergillus flavus* ATCC 26949 in: a) control; b) samples with 0.2 mmol x g^{-1} and c) samples with 0.5 mmol x g^{-1} of investigated coumarine. Cultures were cultivated on maize grains at 28 °C for 21 days, and each point represents the average of two samples.

lated mould growth and aflatoxin accumulation (Table 1). After 21 days of incubation biomass dry weight was higher ca 5% as compared in control, and AFB_1 and AFG_1 were higher 12% and 18.5%, respectively. At a concentration of 0.2 mmol x g^{-1} in 50 g of maize grains, the growth of the mould was reduced by 22% as compared with control and concentration of aflatoxin B_1 was reduced by 30%. After 21 days of cultivation no synthesis of AFG_1 could have been detected (Figure 2b). The biomass content in experiments with 0.5 mmol x g^{-1} of investigated coumarine was lessened. The highest values obtained after 21 days were reduced by 65% as compared in control (Figure 2c). In these experiments the concentration of AFB_1 after 14 days was considerably lower, and no synthesis of AFG_1 could have been detected. The highest concentration of AFB_1 was 54.0 μg x g^{-1} of mycelium dry weight, i.e. about 90% lower than in experiments with 0.2 mmol x g^{-1} of this compound.

In experiments with 1.0 mmol x g^{-1} of coumarine no synthesis of both aflatoxins were found in the samples after seven days of cultivation although biomass content was 9.6 mg x g^{-1} of substrate. After 14 and 21 days the biomass content was 12.4 and 14.2 mg x g^{-1} of substrate, respectively. Concentration of AFB_1 was 170.0 and 65.0 μg x g^{-1} of substrate and no synthesis of AFG_1 could have been detected (Table 1).

In experiments with 2.0 mmol x g^{-1} of this compound there was no synthesis neither biomass nor both aflatoxins after seven and 14 days of cultivation. After 21 days of cultivation small increment of biomass was detected, and no accumulation of both investigated aflatoxins (Table 1)

The data obtained in these investigations are standing in good accordance with the findings of several authors (Sušac *et al.,* 1989; Duraković *et al.,* 2006; Duraković, 2007; Parameshwarappa *et al.,* 2009; Roussaki *et al.,* 2010; Tamura *et al.,* 2010) who have shown that some coumarines strongly inhibit growth of toxigenous moulds and biosynthesis of mycotoxins as aflatoxins.

Our results confirmed that selected investigated coumarine is an effective inhibitor of synthesis of biomass and aflatoxin accumulation in maize contaminated with toxigenous mould *Aspergillus flavus* ATCC 26949.

Conclusions

The purpose of this study was to examine the effectiveness of newly synthesized derivative of coumarine for the control of growth of toxigenous mould *Aspergillus flavus* ATCC 26949 and aflatoxin accumulation on maize grain ZG-SK 502A hybrid. Using different concentrations we found that application of investigated coumarine prior to storage may be a potential means of preventing the growth and aflatoxin accumulation by *Aspergillus flavus* ATCC 26949 in the maize grain. Studies are now being conducted to evaluate the coumarine as antifungal and antimycotoxigenic agent.

These data indicate that on maize grain and under certain conditions, investigated coumarine may provide some antifungal and antimycotoxigenic benefit, and thus health protection against possible aflatoxin accumulation. Further investigations on maize grain hybrids might be useful to study the effectiveness of coumarine on growth and aflatoxin accumulation during the growth of toxigenous mould *Aspergillus flavus* in controlling parameters of cultivation.

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