ORIGINAL SCIENTIFIC PAPER

A Study of Antifungal and Antiaflatoxigenic Action of Newly Synthesized Analogues of Dehydroacetic Acid

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Summary

Mycelial growth and aflatoxin B_1 accumulation by toxigenous mould Aspergillus flavus were inhibited by dehydroacetic acid and its newly synthesized analogues, 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (DHT) and 5-Bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (BrDHT) during the growth of investigated mould on solid substrate (soy-beans).

Experiments were carried out in a stationary culture at 20 ^{0}C and 30 ^{0}C, respectively, during 21 days. Mycelial growth (biomass dry weight) was determined by measuring the chitin content and concentration of aflatoxin B_1 (AFB_1) was quantitated by HPLC. Concentrations of DHA and DHT of 0.1 and 0.2 μg x g^{-1} of substrate were stimulated, but concentrations higher than 0.5 μg x g^{-1} of these compounds, mould growth and AFB_1 accumulation partially inhibited. In experiments with 0.2 μg x g^{-1} of BrDHT mould growth and AFB_1 accumulation were inhibited completely depending on the time and temperature of cultivation.

Keywords: toxigenous moulds, aflatoxin B_1, dehydroacetic acid, chitin, HPLC

Sažetak

Rast micelija i nakupljanje aflatoksina B_1 pomoću toksikogene plijesni Aspergillus flavus inhibirani su pomoću dehidracetne kiseline i njenih novosintetiziranih analoga, 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (DHT) i 5-Bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (BrDHT) u tijeku rasta istraživane plijesni na čvrstom supstratu (zrnima soje).

Eksperimenti su načinjeni u stacionarnom uzgoju pri 20 ^{0}C odnosno 30 ^{0}C, u tijeku 21 dan. Rast micelija (suha tvar biomase) određena je mjerenjem količine hitina, a koncentracija aflatoksina B_1 (AFB_1) određena je kvantitativno pomoću HPLC metode. Koncentracije DHA i DHT od 0.1 i 0.2 μg x g^{-1} supstrata su stimulirane, a koncentracije više od 0.5 μg x g^{-1} tih spojeva, samo su djelomično inhibirale rast plijesni i nakupljanje AFB_1. U eksperimentima s 0.2 μg x g^{-1} BrDHT, rast plijesni i nakupljanje aflatoksina potpuno su inhibirani u ovisnosti o vremenu i temperaturi uzgoja.

Ključne riječi: toksikogene plijesni, aflatoksin B1, dehidracetna kiselina, hitin, HPLC

Introduction

Microbes have a marvellous capacity to produce secondary metabolites which frequently have interesting structure and diverse biological activities. The important beneficial role of certain filamentous fungi in the production cheeses, enzymes, antibiotics and numerous other products has been well documented (ICMSF, 2006.; Murphy et al., 2006.). However, it must not be overlooked that specific filamentous fungi have an even longer history for their detrimental influence to man, animals and plants. The biotechnological hazard of microbes, including fungi, have recently been highlighted (CAST Report, 2003.).

Fungal growth occurs under favourable environmental conditions and it is associated with the production of secondary metabolites, many of which can be hazardous to humans and animals (Kokkonen et al., 2005.).

Foods and feeds are frequently contaminated with moulds during harwesting, 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. The conditions which promote mycotoxin production are usually more restricted than those for mould growth. However, the presence of mycotoxins in food products and the amounts produced depending entirely on the ecological and processing parameters of foodstuff (Filtenborg et al., 1996.). In man and animals fungi can cause infections or mycoses, promote alergic response in sensitised subjects, or poisons with toxic metabolites (Binder, 2007.). Toxicity of fungi to man and animals can arise by mycetismus (mushroom poisoning) or by mycotoxicoses. Mycotoxicoses are poisoning by the ingestion of fungal origin on foods which have been altered or damaged by the growth of toxin producing moulds. The discovery in 1960. of the Turkey X-disease in the United Kingdom can undoubtedly be credited for initiating the present renaissance of interest in mycotoxin research. Mycotoxins are toxic secondary metabolites of moulds belonging essentially to the *Aspergillus, Penicillium* and *Fusarium* genera (Chu, 1991.; Filtenborg et al., 1996.; Murphy et al., 2006.). They can be produced on a wide range of agricultural commodities and under a diverse range of situation. Due to their various toxic effects and their good thermal stability, the presence of mycotoxins in

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foods and feeds is potentially hazardous to the health of both humans and animals. Mycotoxins may further be considered as important environmental pollutants synthesized typically, but allways, on grains, nuts and other plant materials. Humans may be exposed to mycotoxins in two ways: by direct consumption of food stuffs which are contaminated with a mycotoxins, or by consumption of residue containing milk or meat from animals which have ingested mycotoxin contaminated feed.

The aflatoxins, a group of hepatocarcinogenic mycotoxins, produced by imperfect fungi *Aspergillus flavus* (Fries) Link and *Aspergillus parasiticus* (Speare) (Bennett and Christensen, 1983.), became established as the etiological agents. Among several derivatives, aflatoxin B1 (AFB1) is the major and most toxic metabolite (Eaton and Gropman, 1994.; Gourama and Bullerman, 1995.; Wu, 2004.). It is potent carcinogen and, in association with hepatitis B virus, is responsible for many thousand of human deaths per annum, mostly in non-industrialized tropical countries (CAST Report, 2003.; Murphy et al., 2006.).

The prevention of mycotoxin formation on agricultural commodities is essentially a problem of preventing the growth and mycotoxin formation by mycotoxigenic fungi. Various such factors as affecting the antifungal activity of some compounds have been described by Condon and Kuc (1960.), Chichester and Tanner (1968.), Halls and Ayres (1975.), Ahmad and Brannen (1981.), Draković S. et al. (1985., 1987a., 1988., 1989., 1993., 1994., 1995.), Paster et al. (1995.), Draković L. et al. (1999.), Cho and Kang (2000.), Draković L. et al. (2000a., 2000b., 2001., 2004.), Velutti et al. (2004.), Draković L. et al. (2005.), Draković S. et al. (2006.), Draković L. (2007a.), Draković L. et al. (2007b.), Chitrapriya et al. (2008.), Draković S. et al. (2008.), Draković L. et al. (2010.).

However, the use of certain antifungal agents must be viewed with reservation because of ecological problems which may develop later.

In 1947. Coleman and Wolf discovered the antimicrobial action of dehydroacetic acid (DHA). This compound appears to be effective even in a high pH range, but has never acquired great significance because a relatively high toxicity. Since the use of DHA as a food preservative is not permitted in Europe (in the USA this compound is permitted as a food preservative), in the Laboratory of Organic Chemistry Faculty of Food Technology and Biotechnology in Zagreb (Croatia) many analogues of DHA were synthesized as a potential antimicrobial agents (Draković S. et al., 1986., 1989.; Sušnik et al., 1992.; Draković S. et al., 1994.; Draković L. et al., 2001.; Draković S. et al., 2006.; Draković L. et al., 2007b.).

The Laboratory of Microbiology of the same Faculty demonstrated that several DHA analogues inhibit the growth of certain species of bacteria, yeasts and moulds (Draković S. et al., 1986., 1987a., 1989., 1991., 1993. and Draković L. et al., 1999., 2000a., 2000b., 2004., 2007b.). This was the basis of our study, which aimed at evaluating the mould growth and aflatoxin B1 accumulation by Aspergillus flavus ATCC 26949 in soy-bean seed in the presence of some newly synthesized analogues of DHA.

Materials and methods

Assay procedure

Assay of antifungal activity

The antifungal activity of DHA and its newly synthesized derivatives DHT and BrDHT was assayed using two techniques as follows (Draković S. et al., 1994.):

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. Agar medium was poured into dishes, and the content of the dish was mixed well. After allowing the agar to solidify, dishes were prepared using a sterile glass propipete. The various concentrations of investigated compound 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 h at temperature of 25 ^{0}C.

b) Assay in flask cultures: 1 mL of the spore suspension containing cca 10^7 of spores x mL^{-1} of *A. flavus* was inoculated in 250 mL Erlenmayer flasks containing spores and medium. Flasks not containing investigated compound served as a control. Flasks were incubated at 25 ^{0}C for 15 days. Thereafter mycelium was separated from the culture broth by filtration through pre-weighed Whatman No. 1 filtar paper, washed with distilled water, air dried overnight, dried in a hot air oven 105 ^{0}C for 1 hour, and weighed to determine the weight of the mycelia, which was taken as a measure of mould growth. The flasks which did not sign of visual growth were further observed up to a period of 30 days.

Substrate for mould growth and aflatoxin accumulation

The basal substrate used in this study was soy-beans. Fifty grams of seeds were distributed in each 300 mL Erlenmayer flasks and sterile distilled water (about 20 mL x 50 g^{-1}) were added to achieve moisture content of 38 %. The investigated DHA and their analogues DHT and BrDHT were synthesized in the Laboratory of Organic Chemistry of Faculty of Food Technology and Biotechnology in Zagreb, and were dissolved in chloroform at a concentration of 1.0 % and 5 %, respectively (Sušnik et al., 1992.).

Their structures are shown in Figure 1.

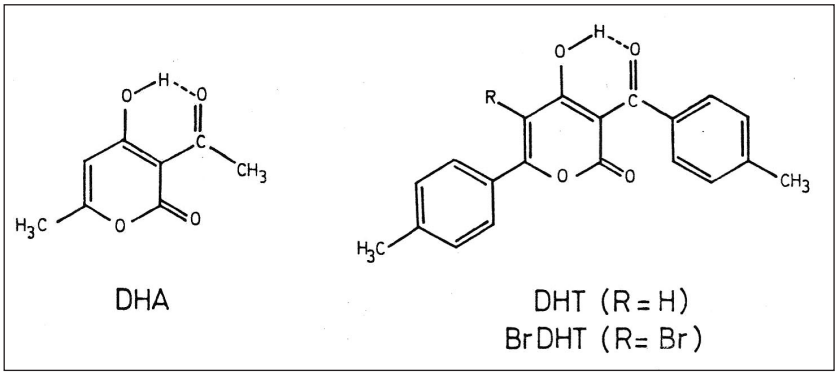

Figure 1. *Chemical structures of Dehydroacetic acid (DHA) and its newly synthesized analogues DHT and BrDHT (Sušnik et al., 1992.; Draković L. et al., 2004.).*

A method for the synthesis of these compounds is described by Sušnik et al., (1992.). Previous work in the Laboratory of Microbiology Faculty of Food Technology and Biotechnology, Zagreb, has demonstrated that several analogues of DHA inhibit the growth of certain species bacteria, yeasts and moulds (Duraković S. et al., 1985., 1989.; Sušnik et al., 1992.; Duraković S. et al., 1994., 1995., 2006.). The above observation was the basis of this study, the objective of which was to evaluate growth and aflatoxin B_1 accumulation by *A. flavus* ATCC 26949 on soy-beans in presence of newly synthesized analogues of DHA.

Culture and inoculum

Aspergillus flavus ATCC 26949 strain was obtained from USDA Fermentation Laboratory Northern Regional Research Center, Peoria, Illinois. The cultures were maintained on slants of potato dextrose agar (PDA) stored at 4 ^{0}C. Before each experiment the mould was transferred to another PDA slant and incubated for 7 days at 25 ^{0}C.

The inoculum for all experiments was prepared in tubes of PDA with conidia of mould incubated for 7 days at 25 ^{0}C. Contents of two tubes, including agar and fungus, were homogenized in 10 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 1 x 10^8 conidia x mL^{-1}.

Treatment of soy-beans with investigated chemical compounds

Soy-beans were slightly cracked, that is, the surface was slightly abraded, but kernels were left whole. This cracking was to allow the mould spores to infect the kernel readily. The beans were cracked after treatment with investigated chemicals but before sterilization by autoclaving. Soy-beans (50 g) were treated with 0.1 to 0.5 µg x g^{-1} of DHA and DHT and with 0.05 to 0.2 µg x g^{-1} of BrDHT, respectively. The test Erlenmayer flasks were kept for 24 hours with through agitation so that the investigated compounds could be absorbed by grains. Flasks not containing analogues served as control. The substrates were then autoclaved at 121 ^{0}C for 20 minutes.

Control test flasks and duplicate test flasks containing the various concentrations of investigated chemicals were inoculated with 1.0 mL of the spore suspension. All the flasks were incubated at 20 ^{0}C and 30 ^{0}C, respectively.

Culture medium and inoculation

Slightly cracked soy-beans were used as media for mould growth and aflatoxin accumulation. The cultivation was performed in stationary cultures with 50 g of substrate in 300 mL Erlenmayer flasks.

Parameters of cultivation were the following:

- Initial water content in the substrate: 38 %
- Initial number of conidia: 2 x 106 per gram of substrate
- Temperature of incubation: 20 ^{0}C and 30 ^{0}C
- Cultivation time: 21 days

Substrates were seeded with 2 x 10^6 spores of investigated mould per gram of substrate. Water content was adjusted by adding appropriate amounts of distilled water to the samples and allowing the liquid and solid phase to equilibrate on a laboratory shaker for 30 minutes.

Biomass determination

Every 7 days control flasks and duplicate test flasks were taken out the incubator as samples for the determination of biomass and AFB1 accumulation. The substrates were autoclaved at 121 ^{0}C for 30 minutes before analysis to kill the spores and vegetative mycelia.

The growth of the fungus was monitored by using the analysis of chitin measured as glucosamine, as a criterion (Donald and Mirocha, 1977.; Duraković S., 1981.).

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 method devises, the polymer is not measured directly but rather is hydrolised 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.

For determining biomass dry weight six different amounts of mould moist mycelium were weighed out during its growth on Sabouraud agar at 25 ^{0}C. The samples were dried at 60 ^{0}C during two hours, and then at 105 ^{0}C to constant mass.

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 S., 1981.).

Duraković S. (1981.) and Duraković L. (2007a.) stated that after 28 days and under comparable environmental conditions, chitin content in mycelium dry weight of mould *Aspergillus flavus* ATCC 26949 was 215 and 230 mg x g^{-1}, respectively.

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

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

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

Detection and measurement of aflatoxin B_1

AFB_1 from all samples was extracted by shaking with 150 mL of chloroform in a Laboratory shaker (50 cycles x min-1 for one hour) at room temperature. The extracts were vacuum filtered and the filtrates evaporated in a flash-evaporator (50 ^{0}C; 1.33 kPa) reducing their volume to about 5 mL. Subsequently, the extracts were purified by column chromatography as reported by AOAC (AOAC International, 2005.).

AFB_1 was identified by thin-layer chromatography (TLC) on silica gel-precoated plates. Separation was achieved with the solvent system chloroform-acetone-petroleum ether (33 : 6 : 1 v/v/v) as described by Duraković S. (1981.) and Duraković

S. et al., (1985.), where after they examined by comparing the R_f values of a known standard to the unknown samples under UV light at 365 nm. Aflatoxin primary standard to check the linearity was examined by Carl Roth (Karlsruhe, Germany). Working standard solutions of 0.25 and 0.125 ng x mL^{-1} of AFB_1 was prepared by diluting the primary standard solution with methanol-acetonitrile (1 : 1 v/v).

After prepartative TLC (Silica gel F_{254}; layer thickness 2 mm), the AFB_1 was assayed quantitatively by HPLC method (AOAC International, 2005.).

HPLC analysis was performed 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 aflatoxin was carried out at $lambda_{ex}$ 360 nm and lambdaem 420 nm. For aflatoxin B_1 the fluorescence detector was set at 360 nm and 420 nm and the mobile phase consisted of a mixture of deionised water-acetonitrile-methanol (60 : 25 : 15 v/v/v). The flow rate was 1 mL x min^{-1} for each mobile phase and the injected volume of working standard was 50 µL.

The percentage inhibition of AFB1 was determined according to the formula (Duraković L., 2007a.):

$$\% \text{ inhibition} = 100 - \left(\frac{\gamma_1}{\gamma_2} \text{ x } 100\right) \quad (2)$$

γ_1 = µg of AFB_1 in substrate containing inhibitor
γ_2 = µg of AFB_1 in control substrate

Results and discussion

The influence of DHA and its DHT and BrDHT analogues of mould growth and AFB_1 accumulation by toxigenic mould *A. flavus* ATCC 26949 is shown in Figures 2-4. Mould growth and accumulation of AFB_1 were observed for 21 days at temperatures 20 ^{0}C and 30 ^{0}C, respectively, after inoculation on sterile soy-beans. According to previous findings (Shindler et al., 1967.; Sorensen et al., 1967. and Duraković S., 1981.) the optimal temperature for the biosynthesis of aflatoxin B_1 is 28-32 ^{0}C.

Figure 2. shows the results of incubation at 20 ^{0}C; only slight growth of *A. flavus* was observed at this temperature. The greatest amount of biomass obtained in controls after 21 days was 4.25 mg dry wt. x g^{-1} of substrate. The amount of AFB_1 is very low, about 0.45 µg x g^{-1} of biomass dry wt. Maximum concentration was established 21 days later for the inoculation. The obtained values are in good accordance with the findings of Buchanan et al. (1981.), Buchanan and Fletcher (1983.) and Duraković L. et al. (2007b.) who showed that the growth of *A. flavus* ATCC 26949 in semisynthetic media and on maize grains at 20 ^{0}C is very low. The data in Figure 2. also shows at the incubation temperature of 30 ^{0}C excellent growth and AFB_1 accumulation in control flasks. The high rate of the accumulation of AFB_1 was observed after 14 days of cultivation and maximum concentration was 16.5 µg x g^{-1} of mycelium dry wt. The highest amount of biomass after 14 days was 13.8 mg x g^{-1} of substrate.

Data on the effect of many chemicals, including fungicides and insecticides, on the inhibition of mycotoxins generated by different fungal species grown on various substrates, have been reported by many researchers (Duraković S. et al., 1984., 1987b., 1989.; Cho and Kang, 2000.; Lavermicocca et al., 2000.; Velutti et al., 2004.; Duraković S. et al., 2006.; Shatzmayr et al., 2006.; Duraković S. et al., 2008.; Parameshrawappa et al., 2009.).

Research by Rao and Harein (1973.) and Duraković S. et al. (1987b.) on *A. parasiticus* NRLL 2999 in YES medium shows the organophosphate insecticide dichlorvos to be an effective antifungal and antiaflatoxigenic agent. According to Chang and Brannen (1975.) mycelial growth and aflatoxin production were totally inhibited by 250 ppm butylated hydroxyanisole.

The influence of DHA on the growth of the same moulds was reported by Webb (1966.). In experiments with moulds

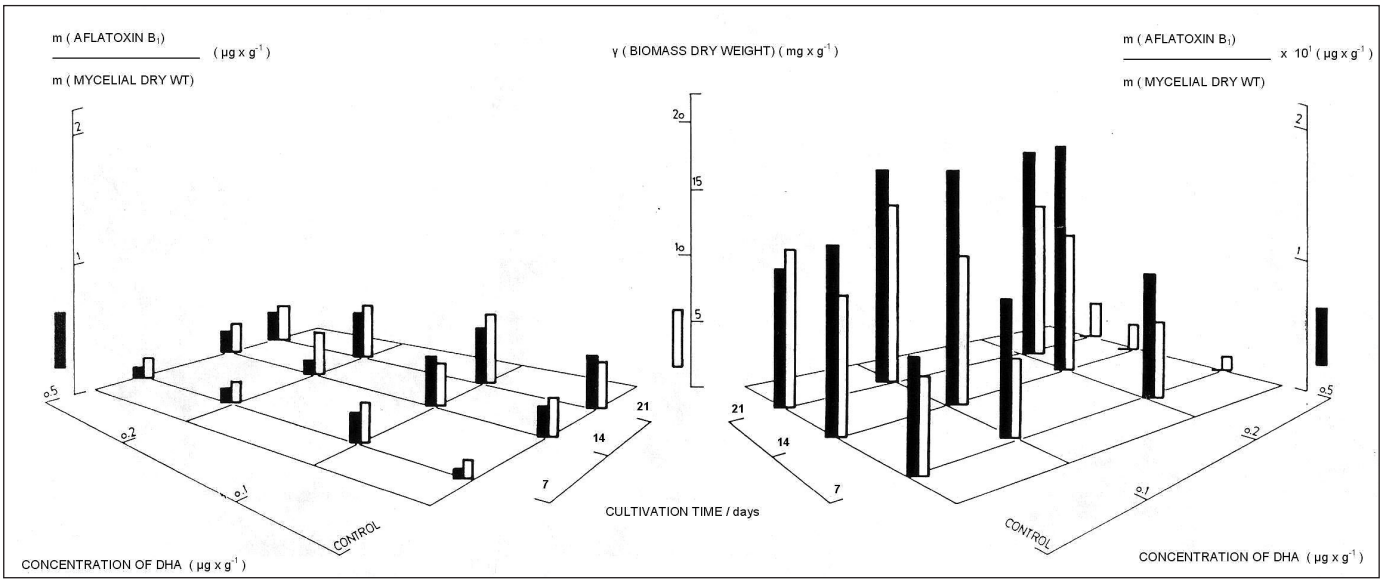

Figure 2. *Comparison of mould biomass and AFB_1 accumulation during the growth of A. flavus ATCC 26949 in the presence of different concentrations of DHA. Cultures were cultivated on soy-beans at 20 ^{0}C (left) and 30 ^{0}C (right) during 21 days.*

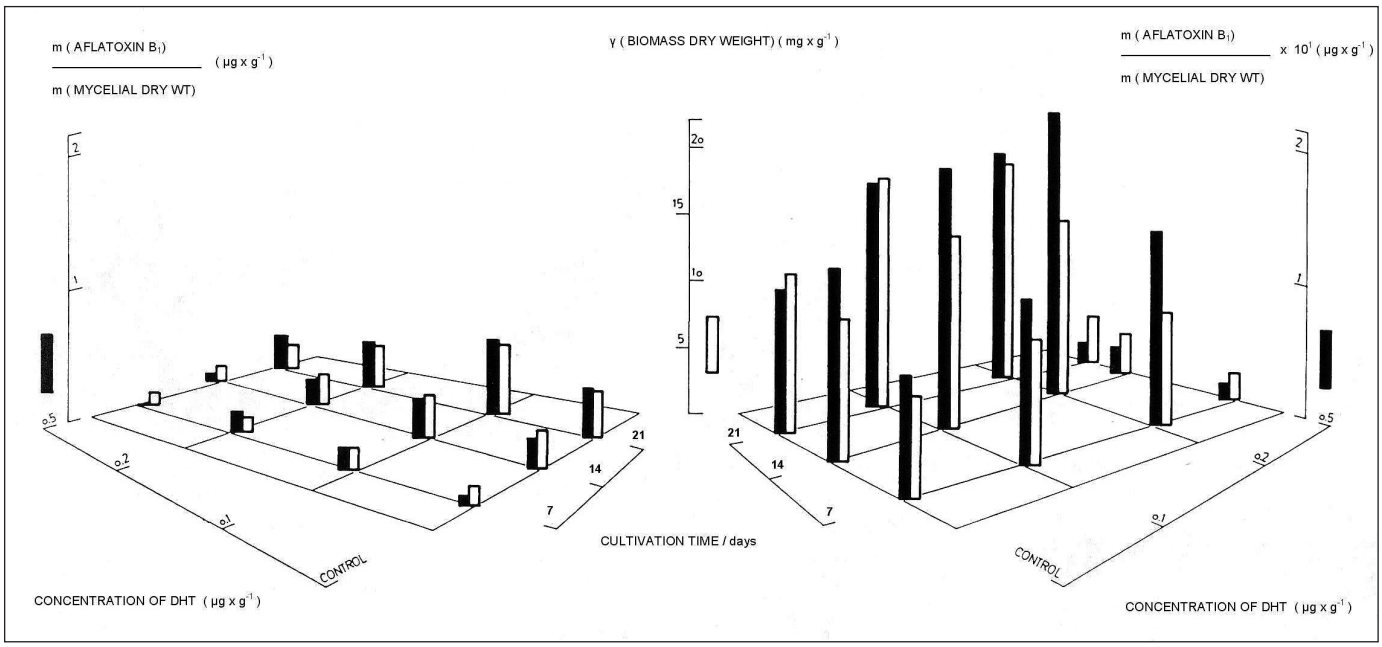

Figure 3. *Comparison of mould biomass and AFB_1 accumulation during the growth of A. flavus ATCC 26949 in the presence of different concentrations of DHT. Cultures were cultivated on soy-beans at 20 ^{0}C (left) and 30 ^{0}C (right) during 21 days.*

from the genus *Aspergillus* and *Penicillium*, the minimal inhibitory concentration was 300 µM. Draković S. et al. (1987b., 1994.) claim that, although DHA acts as an inhibitor of the biosynthesis of certain mycotoxins from certain moulds grown on various substrates, once the synthesis takes place it does not reduce the concentrations of the mycotoxin present.

As shown in Figure 2. DHA at concentrations of 0.1 and 0.2 µg x g^{-1} of substrate produced no inhibitory effect on mould growth and AFB_1 accumulation in experiments at 20 ^{0}C and 30 ^{0}C. Indeed, these concentrations of DHA appeared to have a stimulatory effect on the growth and AFB1 accumulation during 21 days of cultivation. Mould growth was 30 % and 45 % higher and concentration of AFB_1 was 15 % and 25 % higher, respectively, of that in the control. In experiments at 20 ^{0}C, concentration of 0.5 µg x g^{-1} of DHA caused 62 % reduction of growth of *A. flavus*. Accumulation of AFB_1 was 48 % of that in controls. At 30 ^{0}C no accumulation of AFB_1, although a small increment of biomass was observed. Growth of biomass was reduced by 75 % as compared in control experiments.

Figure 3. depicts the effect of DHT on the mould growth and AFB1 accumulation, and effect of temperature and incubation time. At concentration of 0.1 µg x g^{-1} of soy-beans by two investigated temperatures, the growth of the mould and AFB_1 accumulation were stimulated. After 21 days of cultivation, mould growth was 45 % and 60 % higher as compared in controls and concentration of AFB1 was 50 % and 60 % higher, respectively. In experiments at 20 ^{0}C and DHT concentration of 0.2 µg x g^{-1}, mould growth and concentration of AFB1 were almost identically to the concentration in controls (0.40 µg AFB_1 x g^{-1} mycelium dry wt., and 4.2 mg of biomass dry wt. x g^{-1} of soy-beans). The greatest amount of biomass obtained at 30 ^{0}C was 50 % higher as compared with controls. At a DHT concentration of 0.5 µg x g^{-1} of substrate at this temperature, mould growth was reduced by 55 %, and concentration of AFB_1 was reduced by 75 %. At an incubation temperature of 30 ^{0}C the growth of *A. flavus* was reduced by 65 %, and concentration of AFB_1 was reduced by 90 % as compared with the control experiments. The values are in good accordance with the findings of some authors (Draković S. et al., 1988., 1989., 1991., 1994.; Draković L. et al., 1999., 2000a., 2000b., 2004., 2005.; Draković L., 2007a.; Draković L. et al., 2007b.; Prakash et al., 2007.; Chitraprya et al., 2008.; Draković S. et al., 2008.; Rudenko et al., 2009.; Draković L. et al., 2010.). These authors stated 0.2 and 0.5 µg x g^{-1} of solid substrates and 0.5 and 1.0 mmol x L^{-1} of liquid substrates as the minimal concentrations of DHA and DHT for the inhibition of growth and mycotoxin synthesis from genera *Aspergillus, Penicillium, Fusarium* and *Trichothecium*.

The data presented in Figure 4. shows the effect of BrDHT on growth and AFB_1 accumulation by mould *A. flavus* ATCC 26949. The activity of this compound was determined for the concentrations between 0.05 to 0.2 µg x g^{-1} of soy-beans.

Concentrations of 0.05 and 0.1 µg x g^{-1}, respectively, were a reduced both mould growth and AFB_1 accumulation at investigated temperatures of cultivation. In experiments at 20 ^{0}C, these concentrations were a reduced accumulation of AFB_1, after 21 days of cultivation, to 55 % and 30 %, of that in control. The growth of the A. flavus was reduced by 15 % and 35 %, respectively. According to Draković S. et al. (1984., 1986., 1988., 1994.), Draković L. et al. (1999., 2001., 2005.), Draković L. (2007a.) and Draković L. et al. (2007b.) the minimal inhibitory concentration of BrDHT for the inhibition of many different fungal species was 0.05 to 0.2 µg x g^{-1} of substrate. In experiments with 0.2 µg x g^{-1} of BrDHT, the accumulation of AFB_1 was reduced completely, and mould growth was reduced to 15 % of the control values. The biomass content in experiments at 30 ^{0}C with 0.05 and 0.1 µg x g^{-1} of BrDHT was reduced to 30 % and 10 %, respectively, as compared in control, and no accumulation of AFB_1 was detected. As expected, in experiments with 0.2 µg of this compound per gram of soy-beans there was no synthesis either biomass nor AFB1.

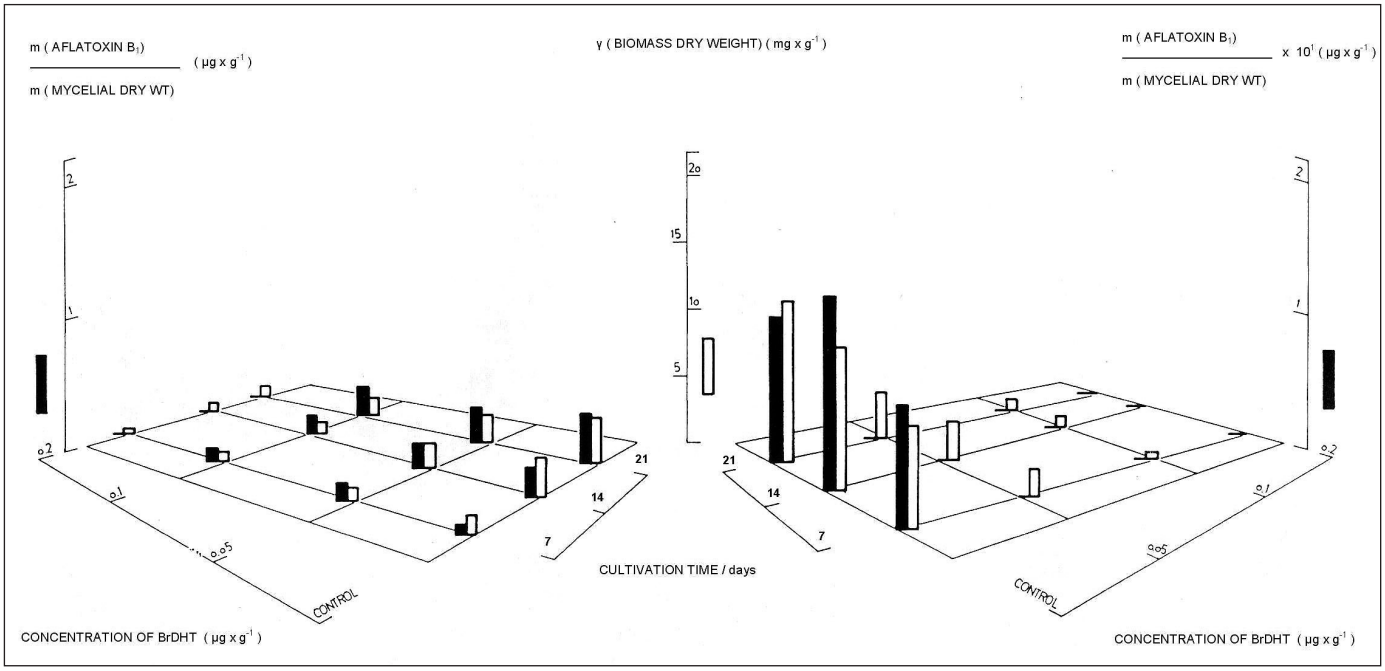

Figure 4. *Comparison of mould biomass and AFB_1 accumulation during the growth of A. flavus ATCC 26949 in the presence of different concentrations of BrDHT. Cultures were cultivated on soy-beans at 20 ^{0}C (left) and 30 ^{0}C (right) during 21 days.*

These data indicated that on soy-beans and under certain conditions, BrDHT may provide some fungistatic and fungicide benefit, and thus health protection against possible aflatoxin development.

Conclusions

Since 1960. the subject of mycotoxicology has developed enormously and attracted much attention from the scientific community. In 2010., on the 50th anniversary of that historic event, there is still no abamtement of that interest.

Many novel mycotoxins were isolated and characterized and mycotoxin research is concerned with toxins that contaminated foods and feeds. These natural toxicants are ubiquitous, knowing no polytical or geographic barriers, and can be credited with catalyzing laudable international scientific collaboration.

The increated interest in biopreservation of food systems has recently led to the development of new synthesized antimicrobial compounds having different origins.

A variety of chemical compounds to prevent food 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, dehydroacetic acid and its newly synthesized derivatives and coumarines (Bullerman et al., 1977.; Duraković S. et al., 1986., 1989., 1994., 1995.; Basilico and Basilico, 1999.; Marin et al., 2003.; Velutti et al., 2004.; Duraković S. et al., 2006., 2008.; Duraković L. et al., 2010.).

We studied dehydroacetic acid and its two newly synthesized analogues used to protect stored grains (soy-beans) namely DHT and BrDHT. Specially, we investigated their effects on AFB_1 accumulation by strain *A. flavus* ATCC 26949.

The data obtained in this work suggest that application of investigated analogues prior to storage may be a potential means of preventing the growth and AFB_1 accumulation by A. flavus ATCC 26949 in soy-beans.

Studies are now being conducted to evaluate the investigated analogues as antifungal and antiaflatoxigenic agents.

It is hoped that this publication makes a contribution to this young and growing science.

Acknowledgements

The authors express their gratitude to Professor E.B. Lillehoy, United States Department of Agriculture, Peoria, Illinois for providing a strain of *Aspergillus flavus*.

We are also grateful to Professor M. Terzić for analyses of aflatoxin in soy-bean and for information.

The investigations were supported by a grant No. 058-0582184-0432 from Croatian Ministry of Science and Technology.

Last, but not least, thanks to gentlewoman Brigitta Duraković for language correcting the manuscript.

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