MOULD GROWTH AND AFLATOXIN ACCUMULATION IN THE PRESENCE OF NEWLY SYNTHETIZED ANALOGUES OF DEHYDROACETIC ACID

K. Tkalčević[1], S. Duraković[1], I. Sušnik-Rybarski[1], Z. Duraković[2], O. Pospišil[1], B. Radić[3] and Lj. Brečević[4]

Laboratory of Microbiology and Laboratory of Organic Chemistry, Faculty of Food Science and Biotechnology, Zagreb[1], Department of Internal Medicine, University Hospital Rebro, Zagreb[2], Institute for Medical Research and Occupational Health, University of Zagreb, Zagreb[3], Ruđer Bošković Institute, Zagreb[4]

Dehydroacetic acid (DHA) and its newly synthetized 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), were tested for antifungal and antiaflatoxigenic acitivity in experiments with the aflatoxigenic mould *Aspergillus parasiticus* NRRL 2999. DHT and DHA concentrations of 3.0 μM and 6.0 μM respectively stimulated mould growth and aflatoxin B_1 and G_1 synthesis, but concentrations higher than 30.0 μM produced an inhibitory effect. In the presence of low BrDHT concentrations mould growth decreased by 70% and toxin concentrations by 85% and 90%, respectively.

Aflatoxin B_1 (AFB_1) and other related difurocoumarine toxins are secondary metabolites produced by the fungal genera *Aspergillus*, *Penicillium* and *Rhizopus* (1). AFB_1 has been associated with certain mycotoxicoses (2, 3) including turkey »X« disease (4), and has been suggested to be carcinogenic (1). Along with the fungi that produce it AFB_1 is found in a number of agricultural commodities (3) and therefore presents a possible risk to human and animal health.

To prevent aflatoxin formation in agricultural commodities it is first necessary to prevent the growth of aflatoxin producing fungi. 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. The use of chemical antifungal agents to control aflatoxin production has been intensively investigated (5–19). 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* (16) discovered the antimicrobial action of DHA. This compound appears to be effective even in a high pH range, but has never acquired great significance because of its relatively high toxicity. As the use of DHA as a food preservative is not permitted in Europe, in the Laboratory of Organic Chemistry of the Faculty of

Food Science and Biotechnology in Zagreb many DHA analogues were synthetized as potential antimicrobial agents. The Laboratory of Microbiology of the same Faculty has demonstrated that several DHA analogues inhibit the growth of certain species of bacteria, yeasts and moulds (17). This was the basis of our study, which aimed at evaluating the mould growth and aflatoxin accumulation by *A. parasiticus* NRRL 2999 in a semisynthetic medium in the presence of newly synthetized DHA analogues.
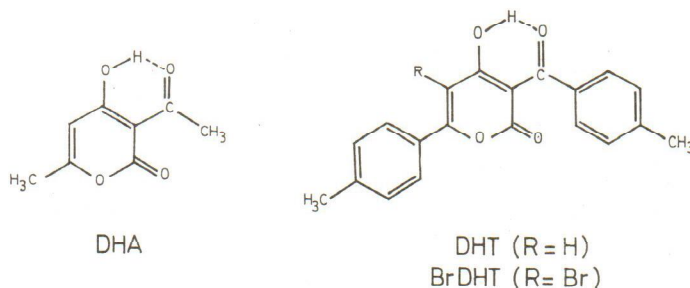

Fig. 1. *Structures of DHA and its newly synthetized analogues DHT and BrDHT.*

MATERIALS AND METHODS

Microorganism

Aspergillus parasiticus NRRL 2999 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 organism was transferred to another PDA slant and incubated at 28°C for seven days.

Inoculum

Inoculum was produced by growing the mould on PDA slants for about seven days at 28°C until it was well sporulated. Inocula were obtained by adding 5 mL of sterile 5 ppm-solution of Triton X-100 and aseptically dislodging the spores with a sterile inoculating loop. The culture was shaken vigorously for 1 min and spore number was determined using a Thoma counting chamber. The spore suspension was diluted to 5×10^7 spores/mL.

Culture medium and inoculations

The basal medium used in this study consisted of 2% yeast extract and 20% sucrose (YES) broth which is known to support aflatoxin production (18). The broth was dispensed in 50 mL aliquots into 250 mL Erlenmeyer flasks. The pII was adjusted with 1 M IICl and 1M NaOH before sterilization. The flasks were autoclaved at 121°C for 20 min. The final pH of the medium was adjusted to 4.5 ($\pm$ 0.2). The inhibitors were dissolved in chloroform at a concentration of 0.001% (19). Their structures are shown in Fig. 1.

The required amounts of inhibitor solutions were pipetted into test Erlenmeyer flasks to give 1.0, 10.0 and 50.0 mg · L^{-1} in 50 mL of broth. Control flasks and duplicate test flasks containing the various concentrations were inoculated with 1.0 mL of the spore suspension of the fungus. All the flasks were incubated at 30°C for 28 days.

Assay of cultures for aflatoxins

The amount of biomass and AFB_1 and AFG_1 concentrations were determined in control flasks and duplicate test flasks every seven days during cultivation. The substrates were autoclaved at 121 °C for 30 min before analysis for aflatoxin, to kill the spores and vegetative mycelia. From all samples aflatoxins were extracted by shaking with 100 mL of chloroform on a laboratory shaker (50 cycles/minute) for one hour at room temperature. The extracts were vacuum-filtered and the filtrates evaporated to about 5 mL in a flash evaporator (50 °C, 1.33 kPa).
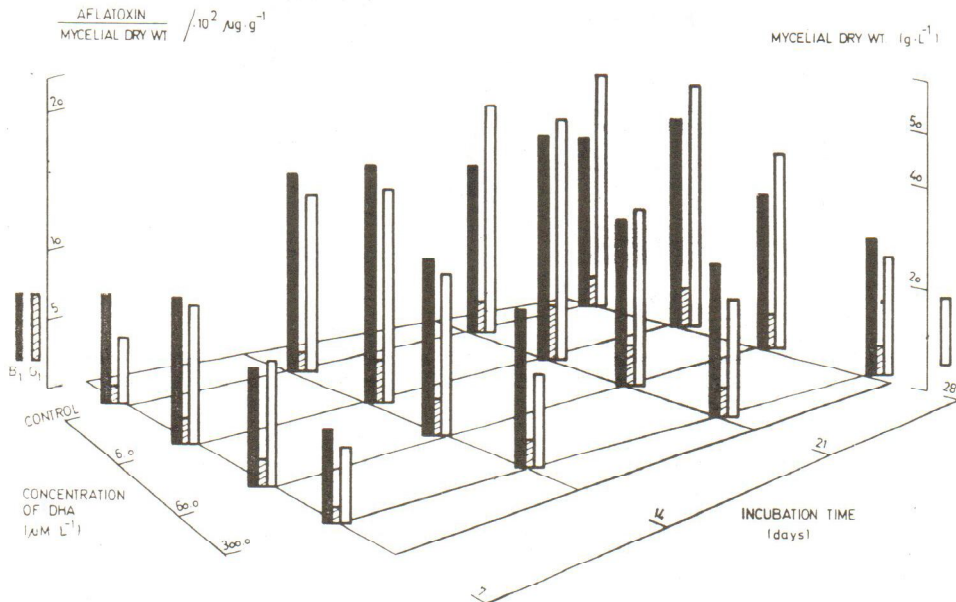

Fig. 2. *Comparative representation of biomass growth and accumulation of aflatoxins during the cultivation of mould A. parasiticus NRRL 2999 in YES medium, in respect to DHA concentration and cultivation time.*

Qualitative and quantitative measurement of aflatoxins

The extracts were purified by column chromatography (20) and the toxins in the concentrated extracts were separated by thin-layer chromatography (TLC) on plates precoated with silica using chloroform-acetone-petroleum ether (33:6:1) as the developing solvent (21). The TLC plates were scanned with a Camag-fluorodensitometer equipped with a W + W – plotter (22–24) having filter excitation and emission settings of 365 and 436 nm respectively. Aflatoxin concentrations in the test samples were calculated from fluorescence intensities which had been compared with those of known concentrations of standard commercial aflatoxins (25). The percentage inhibition of both toxins were determined according to the formula:

$$\% \text{ inhibition} = 100 - (\frac{\mu g \text{ of aflatoxin/mL of broth containing inhibitor}}{\mu g \text{ of aflatoxin/mL in control broth}} \times 100)$$

Fig. 3. *Comparative representation of biomass growth and accumulation of aflatoxins during the cultivation of mould A. parasiticus NRRL 2999 in YES medium, in respect to DHT concentration and cultivation time.*
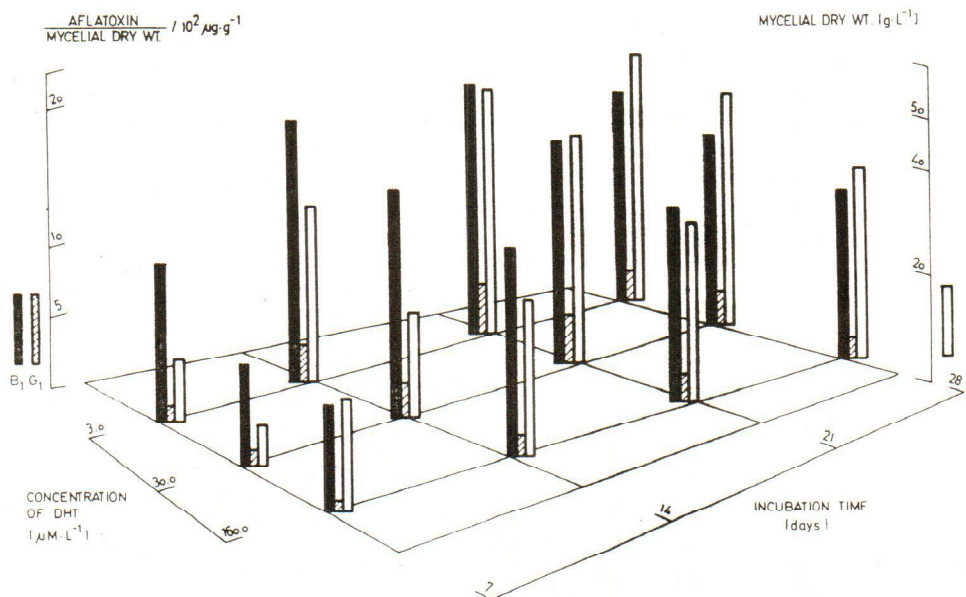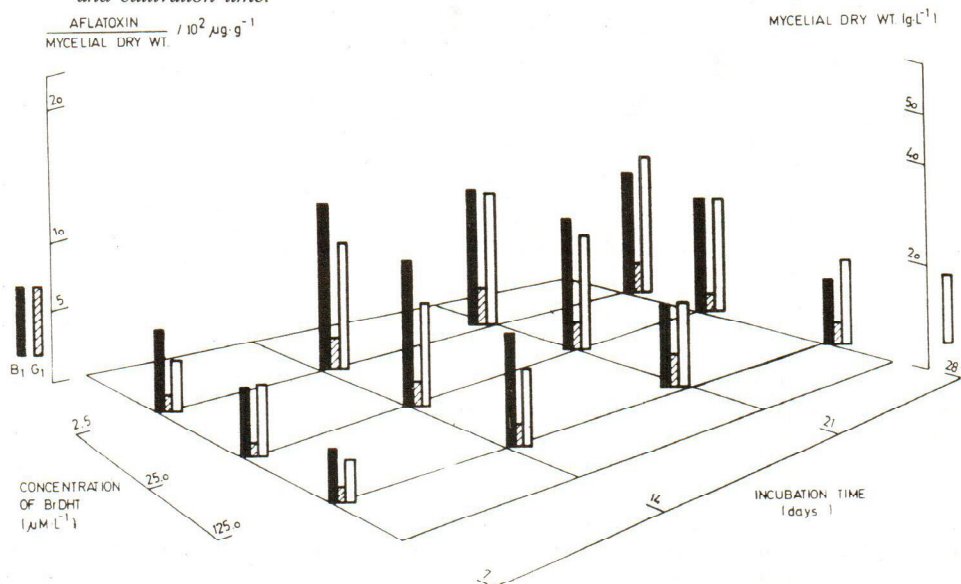

Fig. 4. *Comparative representation of biomass growth and accumulation of aflatoxins during the cultivation of mould A. parasiticus NRRL 2999 in YES medium, in respect to BrDHT concentration and cultivation time.*

Measurement of mould growth

After the aflatoxins had been extracted, the mycelial mats were separated from the medium by filtration through pre-weighed Whatman No 1 filter paper, washed with distilled water, air-dried overnight, dried in a hot-air oven at 105 °C for one hour, and weighed to determine the mycelia as a measure of mould growth. The percentage inhibition of mould growth was determined according to the formula:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{mg of biomass dry weight containing inhibitor}}{\text{mg of biomass dry weight in control sample}} \times 100 \right)$$

RESULTS AND DISCUSSION

The effect of DHA and its DHT and BrDHT analogues on mould growth and AFB_1 and AFG_1 accumulation by the toxigenic mould *A. parasiticus* is shown in Figures 2–4.

Fungal growth and aflatoxin accumulation were observed for four weeks at 30 °C after inoculation of *A. parasiticus* in sterile YES medium. According to *Shindler and co-workers* (7) and *Sorenson and co-workers* (8) the optimal temperature for the biosynthesis of AFB_1 is 28–32 °C, whereas for AFG_1 it is 24–28 °C.

Figure 2 shows excellent mould growth and synthesis of both toxins in control flasks at incubation temperature. In control samples the greatest amount of biomass after four weeks was 44 g dry weight per litre. The high rate of AFB_1 accumulation was observed after two weeks of cultivation, whereas the accumulation of AFG_1 reached its maximum after three weeks. The maximum toxin concentrations were: 1450 μg B_1/g dry weight of mycelium, and 240 μg G_1/g dry weight of mycelium. The values are in good accordance with the findings of *Buchanan and Fletcher* (13) and *Buchanan and co-workers* (14) who showed that the growth rate of *A. parasiticus* NRRL 2999 in AMY and YES media at 28 °C was 30 and 38 mg dry wt/mL. Total aflatoxin concentration in their experiments was 1200 μg/g dry wt of mycelium.

The effects 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 (6–11). Thus *Bullerman* (10) investigated the antifungal properties of cinnamon in media and raisin bread. He reported the spice to inhibit the growth *A. parasiticus* and its aflatoxin production, toxin production being inhibited to a greater extent than mycelial growth. According to *Chang and Branen* (11) mycelial growth and aflatoxin production were totally inhibited by 250 ppm butylated hydroxyanisole. Research by *Rao and Harein* (9) and *Duraković and co-workers* (17) on *A. parasiticus* NRRL 2999 in YES medium shows the organophosphate insecticide dichlorvos to be an effective antifungal and antiaflatoxigenic agent. The effect of DHA on the growth of some moulds was reported by *Webb* (6). In experiments with moulds from the genus *Aspergillus* and *Penicillium*, the minimal inhibitory concentration was 300 μM. *Sušnik--Rybarski and co-workers* (19) claim that, although DHA acts as an inhibitor of the biosynthesis of certain mycotoxins from 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 a concentration of 6.0 μM $\cdot$ L^{-1} produced no inhibitory effect on mould growth and accumulation of AFB_1 or AFG_1 by *A. parasiticus* NRRL 2999. According to *Webb* (6) higher DHA concentrations are necessary for inhibition of

toxigenic fungi. Indeed, this concentration of DHA appeared to have a stimulatory effect on the growth of the investigated mould and on aflatoxin accumulation. DHA concentrations of 60.0 and 300.0 μM caused a 20% and 55% reduction in the growth of *A. parasiticus*. At 60.0 μM of DHA, AFB_1 accumulation was 90% of that in controls, whereas the accumulation of AFG_1 was 15% higher than in controls (Fig. 2). A DHA concentration of 300.0 μM decreased the accumulation of AFB_1 to 85%, and that of AFG_1 to 95% as compared with the results of control experiments.

Figure 3 depicts the effect of DHT on mould growth and aflatoxin accumulation, and the effect of incubation time. At DHT concentrations of 3.0 and 30.0 μM in 50 mL of YES medium the growth of the mould and aflatoxin accumulation were stimulated. Mould growth was 30% higher as compared with the control values and concentrations of both toxins were 20% and 35% higher. The greatest amount of biomass obtained in experiments with a DHT concentration of 30.0 μM was almost identical to that in control experiments but the concentration of both aflatoxins was 8% and 20% higher as compared with the controls. At a DHT concentration of 160.0 μM, mould growth was reduced by 25%, and AFB_1 and AFG_1 concentrations were reduced by 5% and 12%, respectively.

Figure 4 shows the effect of BrDHT on mould growth and on aflatoxin accumulation by the mould. The activity of this compound was determined for the concentrations between 2.5 and 125.0 μM. In experiments with 2.5 and 25.0 μM of BrDHT, the maximum mould growth was 70% and 55% of that in the control YES medium. A 2.5 μM BrDHT concentration reduced the production of both investigated toxins to 92% and 85%, respectively. At 25.0 μM of BrDHT, accumulation of AFB_1 was 80% of that in control experiments, whereas the accumulation of AFG_1 was 75% of the control value. The growth of *A. parasiticus* and accumulation of aflatoxins in the media containing 125.0 μM of BrDHT is shown in Figure 4. The accumulation of aflatoxins was reduced to 45% and 60% of the control values. The growth of mould biomass was reduced by 35%.

CONCLUSIONS

This study indicates that DHA and DHT are poor inhibitors of mould growth and aflatoxin production by *A. parasiticus* NRRL 2999 in YES medium. Very low DHA and DHT concentrations even stimulated mould growth and accumulation of aflatoxins. Inhibition of aflatoxin production was reduced by concentrations higher than 30.0 and 60.0 μM.

The investigated compound BrDHT, significantly inhibited mould growth and accumulation of aflatoxins in all conditions of cultivation of *A. parasiticus* NRRL 2999 in YES medium.

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Sažetak

RAST PLIJESNI I NAKUPLJANJE AFLATOKSINA
U PRISUTNOSTI NOVOSINTETIZIRANIH ANALOGONA DEHIDRACETNE KISELINE

Istražen je utjecaj dehidracetne kiseline (DHA) i njezinih novosintetiziranih analogona, 4-hidroksi-6-(p-tolil)-3-(p-toluil)-2H-piran-2-ona (DHT) i 5-bromo-4-hidroksi-6-(p-tolil)-3-(p-toluil)-2H-piran-2-ona (BrDHT), na rast aflatoksinogene plijesni *Aspergillus parasiticus* NRRL 2999 i nakupljanje aflatoksina. Koncentracije DHA i DHT od 3.0 i 6.0 μM stimuliraju, a koncentracije više od 30.0 μM tih spojeva inhibiraju rast plijesni i proizvodnju aflatoksina. U pokusima s niskim koncentracijama spoja BrDHT, reduciran je rast plijesni do 70%, a koncentracije aflatoksina B_1 i G_1 bile su reducirane do vrijednosti od 85 odnosno do 90% u odnosu na kontrolne pokuse.

Laboratorij za mikrobiologiju i Laboratorij za organsku kemiju,
Prehrambeno-biotehnološki fakultet, Zagreb[1]
Medicinski fakultet, Klinički bolnički centar, Zagreb[2]
Institut za medicinska istraživanja i medicinu rada,
Sveučilišta u Zagrebu, Zagreb[3]
Institut Ruđer Bošković, Zagreb[4]