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Full Length Research Paper

Impact of new synthesized analogues of dehydroacetic acid on growth rate and vomitoxin accumulation by *Fusarium graminearum* under different temperatures in maize hybrid

Lejla Duraković[1]*, Andrea Skelin[2], Sanja Sikora[2], Frane Delaš[1], Mirna Mrkonjić-Fuka[2], Katarina Huić-Babić[2] and Mihaela Blažinkov[2]

[1]Faculty of Food Technology and Biotechnology, Department of Biochemical Engineering, Laboratory for General and Food Microbiology, University of Zagreb, Pierotti street 6, 10000 Zagreb, Croatia.
[2]Faculty of Agriculture, Department of Microbiology, University of Zagreb, Svetošimunska street 25, 10000 Zagreb, Croatia.

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Previous work indicated that some of the new synthesized analogues of dehydroacetic acid (DHA) were inhibitory to the growth of mycotoxin producing moulds and accumulation of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA). The objective of this study was to determine the specific new synthesized chemical compounds that may be effective against mould growth and vomitoxin (deoxynivalenol) (DON) accumulation by *Fusarium graminearum*. The effect of the investigated 3-/2-aminophenylamine-(p-toluoyl)-4-hydroxy-6-(p-tolyl)-2H-pyrane-2-one (Schiff base) and 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (DHT) on growth and DON accumulation were studied using a mould *F. graminearum* ZMPBF 1244 and maize grain hybrid to determine the possible use of these compounds as a mean of controlling DON accumulation. Schiff base was inhibitory at 0.05 and 0.1 µg/g and DHT at 0.5 µg/g of maize grain. The inhibitory effect of these substances was judged to be the inhibition of growth rather than toxin accumulation. When growth occurred after a delay, DON accumulation occurred when the cultures reached secondary metabolism. Given sufficient time, cultures which were inhibited initially, but which subsequently inhibited their growth, produced toxin levels equivalent to the control cultures. Levels of the Schiff base above 0.2 µg/g almost completely inhibited mould growth or permitted only a small amount of growth that never reached secondary metabolism and never produced DON during the time of this study.

Key words: *Fusarium graminearum*, vomitoxin, Schiff base, chitin, *Artemia salina.*

INTRODUCTION

Fungi cause significant destruction of grain during storage, rendering it unfit for human consumption by decreasing their nutritive value and sometimes by producing mycotoxins. Invasion of cereal grains by fungi is frequently associated with a substantial risk of contamination by mycotoxins.

The dangerous implications of the mycotoxin problem became evident during the Second World War in Russia; humans and horses suffered immensely from alimentary toxic aleukia and stachybotrytoxicosis, respectively. Mycotoxins received full scientific recognition after the implications of the aflatoxin problem became known. We have indeed progressed since Forgacs (1962) refered to mycotoxicoses as the most unfamiliar and least investigated diseases.

Humans may be exposed to mycotoxin in two ways: by direct consumption of foodstuffs which are contaminated with a mycotoxin, or consumption of reside-containing milk or meat from animals which have ingested

*Corresponding author. E-mail: Lejla.Durakovic@pbf.hr. Tel: +385 1 4605-045. Fax: +385 1 4836-424.

mycotoxin-contaminated feed.

Intense investigations of the characteristics of the secondary metabolites with the potential to cause the diseases led to the assessment of the role of mycotoxins, such as trichothecenes and fumonisins in some mycotoxicoses.

The genus *Fusarium* contains various fungal species that can produce mycotoxins both in the field and during storage and that have been implicated in several human and animal mycotoxicoses.

Trichothecenes are a group of skin irritant and inflammatory metabolites and are responsible for mycotoxicoses in farm animals characterized by dermal necrosis, hemorrhagic syndrome, emesis, food refusal and bone marrow depressions (Marasas et al., 1984; Rheeder et al., 1992; Beardall and Miller, 1994).

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxitrichotec-9-ene-8-one), also known as vomitoxin is produced by certain species of *Fusarium* that invade grains in the field and storage.

Fusarium graminearum Schwabe / teleomorph *Giberella zeae* (Schweinitz) Petch, is one of the most destructive species of *Fusarium*, responsible for *Fusarium* head blight or scab of wheat and other small cereal grains worldwide (Placinta et al., 1999; Wendels, 2000; Schollenberger et al., 2002; Lombaert et al., 2003; He-Ping et al., 2005; Schollenberger et al., 2005; Blažinkov et al., 2008; Duraković et al., 2008).

Mycotoxins produced by *F. graminearum* were found on many occasions in food and with trichothecene deoxynivalenol (DON) being the most abundant (Rotter et al., 1996; Schaffsma et al., 2001; Schollenberger et al., 2002). Outbreaks of human intoxication associated with wheat or maize infected by *F. graminearum* were reported from many countries (Marasas et al., 1984; Rheeder et al., 1992; Nelson et al., 1993; Jestoi, 2008). Recent documentation on the frequency of occurrence of DON (Rheeder et al., 1992) suggests that this toxin and other trichothecenes may cause greater health problems than aflatoxin in animals.

Approximately 180 trichothecenes are known to exist but only a few are significant to human health. This summary focuses on DON, the most prevalent of trichothecenes in human foods. Among the fungi producing trichothecenes such as DON, the species *F. graminearum* were predominantly strains, which are widely distributed in the crop field (Creppy, 2002; Edwards, 2004; Ayalew et al., 2006; Al-Hazmi, 2011).

In recent years, legislation has adopted the problem of DON in food. About 60% European wheat is found contaminated with DON at concentrations ranging from 2 to 3960 µg/kg (Gareis et al., 1989). Maximum limits were established in the European Union in February 2002 (European Commission, 2002). They range from 200 µg/kg for cereal products for young children to 1750 µg/kg for unprocessed durum wheat and oats (Directorate General Health and Consumer Protection, 2003). Germany implemented more stringent maximum limits for DON in 2004 ranging from 100 µg/kg for infant cereal products to 500 µg/kg for most other cereal products (Commission Regulation (EC), 2005). Most studies on trichothecenes focus on their incidence in different substrates (DeNijs et al., 1996; Mahnine et al., 2011), identification responsible for their production and their location in cars and kernel fractions (Wetter et al., 1999). A few reports deal with the effect of environmental factors in these mycotoxins accumulation (Birzele et al., 2000).

Some fungicides and new synthesized analogues of dehydroacetic acid and tetraketone have been found to reduce chosen trichothecenes accumulation by *F. graminearum* in synthetic media (Hasan, 1993; Duraković et al., 1995, 2000), but few studies report the chemical treatments for DON prevention. Velutti et al. (2003, 2004) and Marin et al. (2004) found that cinnamon, clove, lemongrass, oregano and palmarosa essential oils were able to inhibit DON synthesis under certain environmental conditions in sterile maize inoculated with *F. graminearum*.

To prevent DON accumulation in agricultural commodities, it is first necessary to prevent the growth of mycotoxin producing fungi. The use of chemical antifungal agents to control DON accumulation has been intensively investigated (Bullerman et al. 1977; Duraković et al., 1995; Paster et al., 1995; Cho and Kang, 2000; Duraković et al., 2000; Velutti et al., 2003).

However, the use of certain antifungal agents must be viewed with reservation because of ecological problems which may develop later. In the Laboratory of Organic Chemistry, Faculty of Food Technology and Biotechnology in Zagreb many analogues of dehydroacetic acid (DHA) and tetraketones and coumarines were synthesized as potentially antimicrobial agents. Methods for the synthesis of these compounds are described by Filipović-Marinić and Laćan (1982), Sušac et al. (1989), Sušnik et al. (1992), Govori et al. (2004), Prakash et al. (2007) and Amanullah et al. (2011).

Preliminary studies in the Laboratory of Microbiology of the same faculty demonstrated that several of these new synthesized compounds strongly inhibit the growth of certain species of bacteria, yeasts and moulds, including trichothecene producing and aflatoxin and ochratoxin producing fungi (Duraković et al., 1986, 1994, 1995, 2006). In experiments with toxigenous moulds *Aspergillus ochraceus*, *Aspergillus flavus* and *F. graminearum,* the authors (Duraković et al., 1987, 1989, 1994, 2004, 2006; Duraković, 2007; Duraković et al., 2010a) showed that some of the new synthesized DHA analogues strongly inhibit growth of these moulds and aflatoxin B_1 and ochratoxin A accumulation in chosen cereals.

Our former investigations included many new synthesized chemical compounds, for example, tetraketones, coumarines and analogues of DHA as

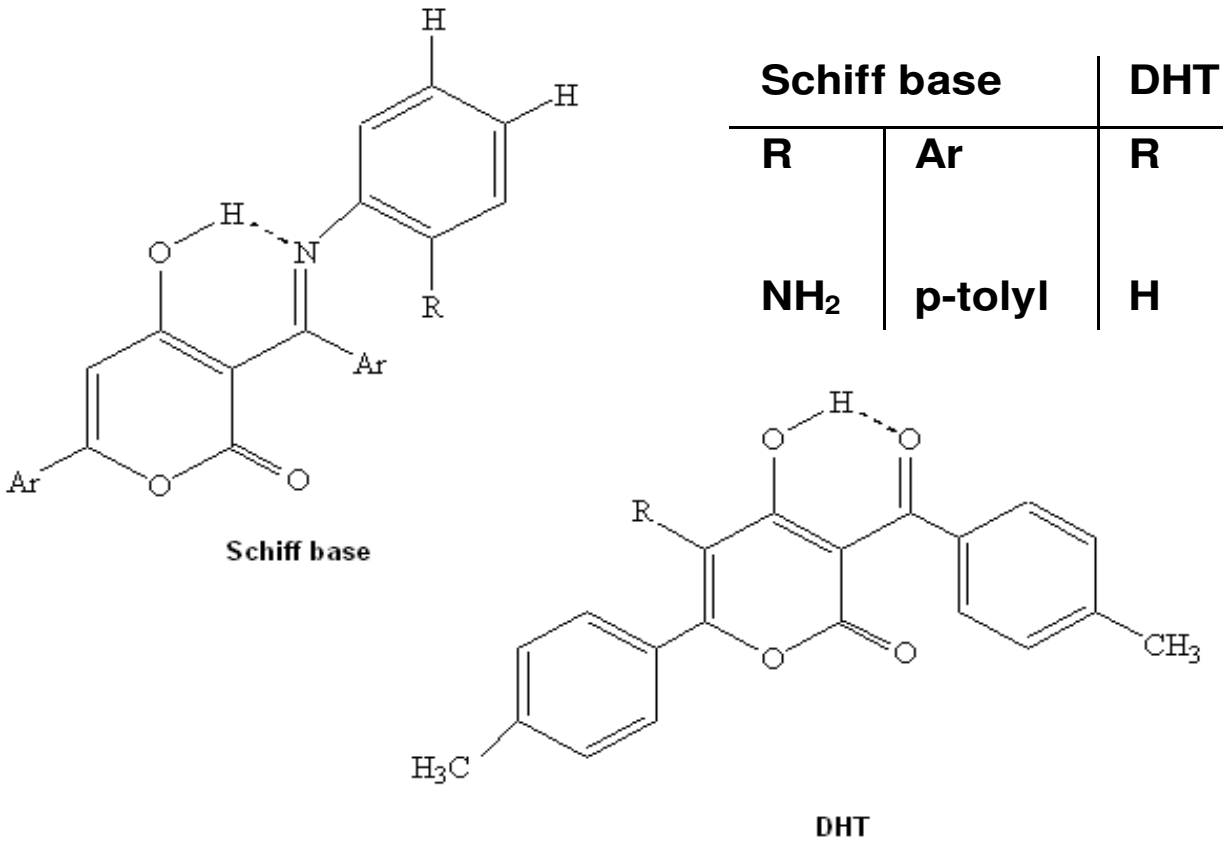

Figure 1. Structures of Schiff base and DHT (Sušnik et al., 1992; Duraković et al., 1994).

potentially antifungal and antimycotoxigenic agents (Sušac et al., 1989; Sušnik et al., 1992; Duraković et al., 1994, 1995, 2010b).

These compounds were synthesized in Laboratory of Organic Chemistry, Faculty of Food Technology and Biotechnology, University of Zagreb. Two of these compounds with circumstantial evidence minimal inhibitory concentration (MIC) of 0.1 and 0.2 µg/ml used in these experiments were DHA analogues 3-/2-aminophenylimino-(p-toluoyl)-4-hydroxy-6-(p-tolil)-2H-pyrane-2-one (Schiff base) and 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (DHT). Their structures are presented in Figure 1.

The objective of this investigation was to assess the convenience of treating maize grain with the new synthesized analogues of DHA (Schiff base and DHT) in order to prevent DON accumulation when inoculated with *F. graminearum* conidia. This *in vitro* assay was based on sterilized maize grain (FAO 280 Os 298 P hybrid) in controlling the parameters of cultivation.

Many biological important Schiff bases ligands have been reported which possess antibacterial and antifungal activity (Raman et al., 2003; Verma et al., 2004; Janos and Tamas, 2009; Zhong et al., 2009; Shi et al., 2010). In order to broaden the scale of investigations on the Schiff bases, we synthesized, structurally characterized and determined antifungal and antibacterial activity of a number of Schiff bases derived from various aromatic aldehydes and aromatic amines and the results will be published.

MATERIALS AND METHODS

Microorganism

F. graminearum ZMPBF 1244, a stable trichothecene vomitoxin (DON) and zearalenone producing strain, was obtained from the Laboratory of Microbiology, Faculty of Food Technology and Biotechnology, Zagreb. The mould was maintained on slants of potato dextrose agar (PDA) stored at 4°C. Before each experiment, the mould was transferred to another PDA slant and incubated at ambient temperature. Figure 2 represents the macroconidia (a) and branched monophialides (b, c and d) of *F. graminearum* which was used in these investigations.

Preparation of inoculum

Potato dextrose agar slants, as the medium for sporulation, were inoculated with conidia of pure mould culture. After incubation at 25°C, the inocula were obtained by adding 5 ml of sterile 5 ppm-solution Triton X-100 in distilled water to 8 days old slants and dislodging conidia with sterile loop under aseptic conditions. The suspension was then adjusted to approximately 5 x 10^8 conidia/ml. Spore number was determined using a Thoma counting chamber.

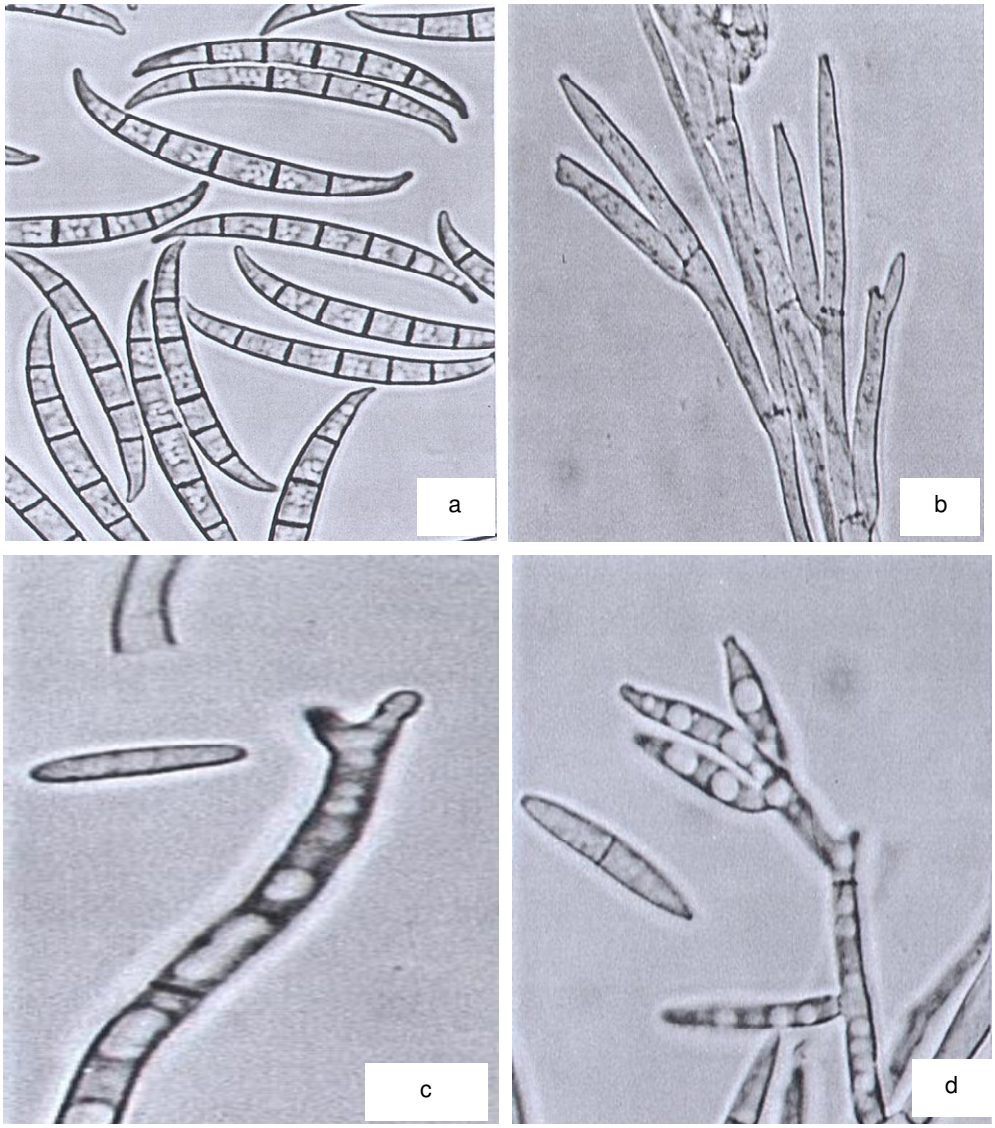

Figure 2. *F. graminearum* ZMPBF 1244. a, macroconidia; b, c and d, conidiophores (unbranched and branched monophialides) (X1000).

Each 50 g of the substrate was inoculated with 1 ml of the diluted suspension.

Assay of antifungal activity

The antifungal activity of Schiff base and DHT was assayed using two techniques as follows (Both, 1972; Duraković et al., 1994):

Standard assay

1 ml of the spore suspension of *F. graminearum* ZMPBF 1244 was used to seed the Petri dishes; prepared by pouring 20 ml of maize extract agar. Agar medium was poured into dishes and the content of the dish were mixed well. After allowing the agar to solidify, dishes were prepared using a sterile glass tube and propipette. 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 for 45 h at 25°C

Assay in flask culture

1 ml of the spore suspension containing cca 10^8 of conidia of *F. graminearum* ZMPBF 1244 was inoculated in 250 ml Erlenmeyer flasks containing 50 g of sterilized maize grains.

On the basis of preliminary information obtained from the dishes assay, appropriate quantities of various concentrations of DHA and Schiff base were added in triplicate to the flasks containing maize grains and spores. The flasks were kept for 24 h with thorough agitation so that investigated compounds could be absorbed by grains. Flask not containing DHT and Schiff base served as the control. Flasks were incubated at 25°C for 15 days. A method of measuring the degree of fungal invasion on corn was the analysis of chitin (Donald and Mirocha, 1977; Duraković, 1981, 2007). The

flasks which did not sign visual growth were further observed up to a period of 30 days.

Substrate for mould growth and DON accumulation

The basal substrate used in this study was maize grains (FAO 280 Os 298 P). Maize grains were slightly cracked, that is, the surface was slightly abraded, but kernels were left whole. This cracking allowed the mould spores to infect the kernel readily. The beans were cracked after treatment with investigated chemicals but before sterilization by autoclaving. The maize grains contained no fungal infection and DON contamination.

50 g of grains were distributed in each 300 ml Erlenmeyer flasks. Sterilized distilled water (about 20 ml/50 g) was added to achieve moisture content of 40%. The investigated Schiff base and DHT were synthesized in the Laboratory of Organic Chemistry of Faculty of Food Technology and Biotechnology in Zagreb, Croatia and were dissolved in chloroform at a concentration of 0.01 and 0.05%, respectively. A method for the synthesis of these compounds is described by Sušnik et al. (1992), Govori et al. (2002) and Chen et al. (2010). The required amounts of these solutions to give 0.05; 0.1; 0.5; 1.0 and 2.0 µg/g were pipetted into test Erlenmeyer flasks. The flasks were kept for 24 h with thorough agitation so that the compounds could be absorbed by grains. Flasks not containing investigated compounds served as control. The substrates were then autoclaved at 121°C for 20 min. Control test flasks and duplicate test flasks containing the various concentrations of investigated chemicals were then inoculated with 1.0 ml of the spore suspension (5 x 10^8 conidia/ml). All the flasks were incubated at a constant temperature of 20, 25 and 35°C for 42 days. Cultures were examined for mould growth and DON accumulation at incubation time of 7, 21, 35 and 49 days.

Measurement of fungal growth

Each 14 days during the cultivation period, control flasks and duplicate test flasks were taken out of the incubator as samples for the determination of mould biomass and DON accumulation. The substrates were autoclaved at 121°C for 30 min to kill the conidia and vegetative mycelia. The growth of *F. graminearum* was monitored by using the analysis of chitin measured as glucosamine; as a criterion (Donald and Mirocha, 1977; Duraković, 1981, 2007; Xiao-E et al., 2008).

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 material occurs in sound cereal grains. In the analytical method devises, the polymer is not measured directly but rather is hydrolyzed 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°C. The samples were dried at 60°C for 2 h and then at 105°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 the amount of biomass dry weight (Duraković, 1981).

Duraković (1981) and Duraković (2007) stated that after 28 days and under comparable environmental conditions, chitin content in mycelium dry weight of mould *F. graminearum* ZMPBF 1244 was 215 and 230 mg/g, respectively. The chitin content of sound maize grain hybrids differed radically from each other, even when they are incubated under similar environmental conditions.

Duraković (1981, 2007) and Duraković et al. (1994, 2010b) stated that after 28 days under comparable environmental conditions, chitin values of 0.125 and 0.190 µg/g were obtained for different maize hybrid, respectively.

Detection of toxicity of investigated DHA analogues

The toxicity of new synthesized DHT and Schiff base were evaluated by using brine shrimp (*Artemia salina)* larvae as a screening system for the determination of their sensitivity to some chemical agents. The lethality test was performed using the method of Harwing and Scott (1971) and McLaughlin et al. (1991). The brine shrimp eggs were hatched in artificial seawater of normal seawater salinity (35 g sea salt per liter of water) and after an average of 24 h from hatching, the shrimp larvae were used for experimental bioassay. For each experiment, 100 to 200 mg of brine shrimp was placed in 100 ml of hatching medium contained in a 500 ml Erlenmeyer flask and shaken as described by Favilla et al. (2006). Hatching can occur in less than 24 h at 27°C.

Results of 10 parallel experiments showed that concentration resulting in 50% mortality of larvae exposed to investigated chemicals for 24 h at 30°C were (µg/ml): Schiff base, 18.50 and DHT, 7.30.

The results suggest that the investigated DHA and Schiff base had a poor toxic effect in relation to *A. salina* larvae in selected cultivation parameters.

Mycotoxin assay

DON determination

DON analysis was carried out according to the protocol recommended by Cahill et al. (1999): DON was extracted with 150 ml of acetonitrile/water (84:16 v/v) added to 50 g of corn, followed by shaking on a Laboratory shaker for 2 h. The extracts were filtered through Whatman No. 1 filter paper. Purification of the crude extract (5 ml) was carried out using a one-step cleanup column [Myco Sep No. 227, Romer Labs. Inc., (Union, MO, USA; method No. LC 1003.2)]. Its limit to detection is 0.1 µg/g. 2 ml of the purified extract was transferred to a vial and evaporated to dryness in a 60°C water bath under vacuum. Residues were re-dissolved in 1 ml of mobile phase, acetonitrile/methanol/water (6:6:88 v/v/v) prior to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses.

After preparative TLC (silica gel F_{254}; layer thickness 2 mm), the DON analysis was performed by using a Hewlett-Packard liquid chromatography (pump and injection system), (Walborn, Germany) with JASCO FP-920 fluorescence detector. The analytical column was C_{18} RP column (250 x 4.6 mm), with particle size of 5 µm (Waters, Inc. Milford, USA).

50 µl eluate samples were injected into the HPLC system. The flow rate of mobile phase was 1.0 ml/min. The wavelength of the absorbance detector was set at 220 nm.

Quantification of DON was accomplished by determination of the area under the chromatographic peak and calculation of the level of DON on the basis of the calibration curve generated with DON standard. The unknown concentrations in the sample were obtained by interpolation in the calibration curve. The limit of detection was 0.1 µg/kg of maize grain. Toxin recovery was determined by spiking DON standard at levels 100, 500 and 1000 ng/kg to control the samples of corn. Recovery averages were found to be in the range of 67 to 97%.

To confirm the identity of DON, TLC method was used (Trenholm et al., 1981). Plates [(Silica gel F_{254}), 250 µm thick, Merck, Darmstadt] were activated at 115°C for 30 min before use. Plates were developed in chloroform/methanol (90:10) and then sprayed with anisaldehyde to detect DON. DON primary standard to check the linearity was examined by Carl Roth (Karlsruhe, Germany).

The change of DON mass fraction (%) was determined according to the formula:

$$\text{Concentration change} = \left(1 - \frac{\gamma_1}{\gamma_2}\right) \times 100$$

Where, γ_1 is the mass fraction of DON in substrate containing inhibitor (µg/g); γ_2 is the mass fraction of DON in the control substrate (µg/g).

Statistical analysis

DON recoveries from the corn samples were performed by using the method recommended by AOAC (1995).

Repeatability and recovery were determined by spiking 50 g of corn with toxin standard solution at the level of 100 to 1000 ng/g prior to the addition of solvent and extraction. After 1 h, DON was extracted from spiked sample and quantified according to the protocol of Cahill et al. (1999). Recovery rates of duplicate experiments were between 85.4 and 89.5, and standard deviation (n= 5) was between 5.3 and 12.1, respectively (Table 1).

There was no significant difference in the mean percent recoveries. The results of toxin investigated were not corrected for recovery.

RESULTS AND DISCUSSION

The influence of DHA analogues Schiff base and DHT on growth and vomitoxin (DON) accumulation by toxigenic mould *F. graminearum* ZMPBF 1244 is shown in Figures 3 to 6 and Tables 2 to 5. Mould growth and DON accumulation were observed for 49 days at 20 and 30°C after inoculation of *F. graminearum* in maize grain. According to previous findings (Beardall and Miller, 1994; Schaafsma et al., 2001; Jestoi, 2008) the optimal temperature for the biosynthesis of DON is 20 to 28°C.

Effect of investigated Schiff base and DHT on mould growth and DON accumulation

In general, higher amounts of DON were found at 20°C than at 30°C, although in the controls, it was higher at 30°C than at 20°C (Tables 2 to 5; Figures 3 to 6).

Figures 3 and 4 and Tables 2 and 3 depict the effect of DHT on the mould growth and DON accumulation and the effect of temperature and incubation time. The high rate of the accumulation of DON was observed after 35 days of cultivation. At a concentration of 0.1 µg/g in maize grain and at the two investigated temperatures, the growth of the mould and DON accumulation were stimulated. After 35 days of incubation, mould growth was 40 and 55% higher when compared with the results obtained in the controls and concentration of DON was 35 and 40% higher, respectively. In the experiments at 20°C and DHT concentration of 0.2 µg/g, mould growth and concentration of DON were almost identical or less stimulated when compared with the controls (57.85 and 55.35 µg DON/g mycelium dry weight and 25.7 and 23.6 mg biomass dry weight/g maize (Figure 3 and Table 2). The percentage inhibition of mould growth was determined according to the formula:

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

Where, m_1 is the biomass dry weight in the flask containing inhibitor; m_2 is the biomass dry weight in the control flask.

At a DHT concentration of 0.5 µg/g and temperature of 20°C, mould growth was reduced by 75% and the concentration of DON was reduced by 30%. At an incubation temperature of 30°C, the growth of the mould was reduced by 65% and the concentration of DON was reduced by 85% when compared with the controls (Figure 4 and Table 3).

The values are in good accordance with the findings of Duraković et al. (1986, 1989, 1994, 2010b) who stated 0.5 µg/g as the minimal concentration of DHT for the inhibition of moulds from the genus *Aspergillus*, *Trichothecium* and *Fusarium*.

The data represented in Tables 4 and 5, and Figures 5 and 6 show the effect of the investigated Schiff base on growth and DON accumulation by the mould. The activity of this compound was determined for the concentrations between 0.05 to 0.2 µg/g. 0.05 and 0.1 µg/g Schiff base reduced the accumulation of DON to 55 and 45%, respectively at 20°C in respect to the highest values obtained after 35 days in the control experiments. The maximum mould growth was 68 and 40%, respectively of that in the control. According to Duraković et al. (1994) and Pandeya et al. (1999), the minimal inhibitory concentration of Schiff base for the inhibition of many fungal species is 0.05 to 1.0 µg/g. In the experiments with 0.2 µg/g of Schiff base, the accumulation of DON was reduced almost completely and mould growth was reduced to 25% of the control values (Table 4; Figure 5). The biomass content at 30°C with 0.05 and 0.1 µg/g of Schiff base was reduced to 35 and 12%, respectively when compared with the controls and the accumulation of DON was reduced to 33 and 5%. As expected, in the experiments with 0.2 µg/g of Schiff base, there was no synthesis neither biomass nor DON (Table 5; Figure 6). These data indicate that in maize grain and under certain conditions, the investigated Schiff base may provide some fungistatic and fungicide benefit and thus, health protection against possible vomitoxin (DON) development.

Conclusions

The increased interest in biopreservation of food systems has recently led to the development of new synthesized

Table 1. Percent DON recoveries from spiked corn samples.

Level spiked (µg/g)	Recovery (%)					Average recovery (%) ± SD
0.1	67	88	96	81	96	85.4 ± 12.1
0.5	84	90	86	97	91	89.5 ± 5.3
1.0	78	94	81	92	93	87.6 ± 7.8

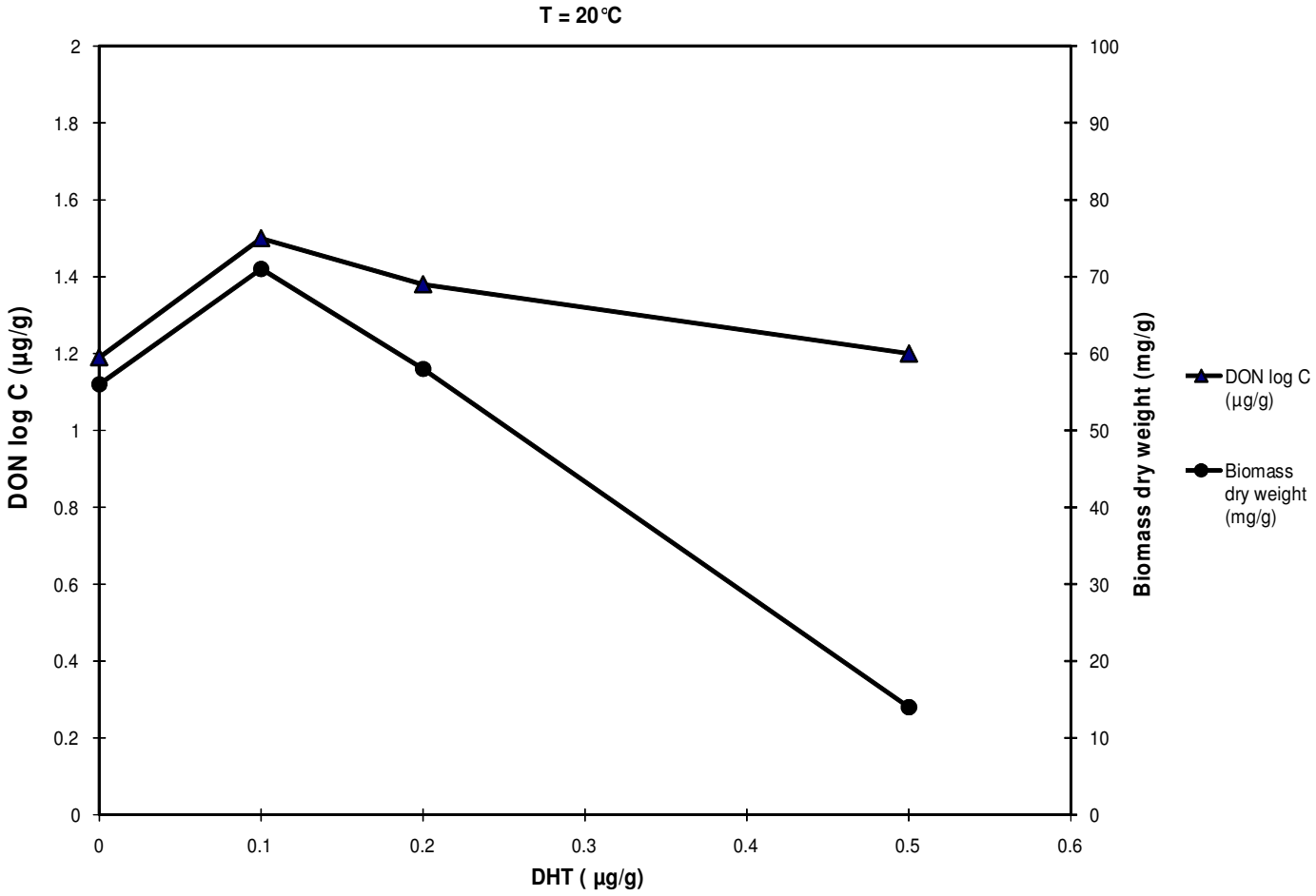

Figure 3. Comparative representation of biomass growth and DON accumulation during the cultivation of mould *F. graminearum* ZMPBF 1244 in the presence of different concentrations of DHT. Cultures were cultivated in maize grain at 20°C (measured after 35 days in relation to values obtained in the control).

antimicrobial compounds having different origins. Among them, dehydroacetic acid (DHA) and its new synthesized analogues are effective antifungal and antimycotoxigenic agents.

The purpose of this study was to examine the effectiveness of the new synthesized analogues of DHA (Schiff base and DHT) for the control of growth of toxigenous mould *F. graminearum* ZMPBF 1244 and accumulation of vomitoxin (DON) in maize grain (FAO 280 Os 298 P hybrid) in selected parameters ofcultivation. Using different concentrations of Schiff base and DHT, we found that Schiff base was the better candidate for the inhibition of mould growth and DON accumulation.

Application of Schiff base prior storage may be acultivation. Using different concentrations of Schiff base and DHT, we found that Schiff base was the better candidate for the inhibition of mould growth and DON accumulation.

Application of Schiff base prior storage may be a potential means of preventing the growth and DON accumulation by *F. graminearum* in the investigated maize grain hybrid. Studies are now being conducted to evaluate the Schiff base as antifungal and

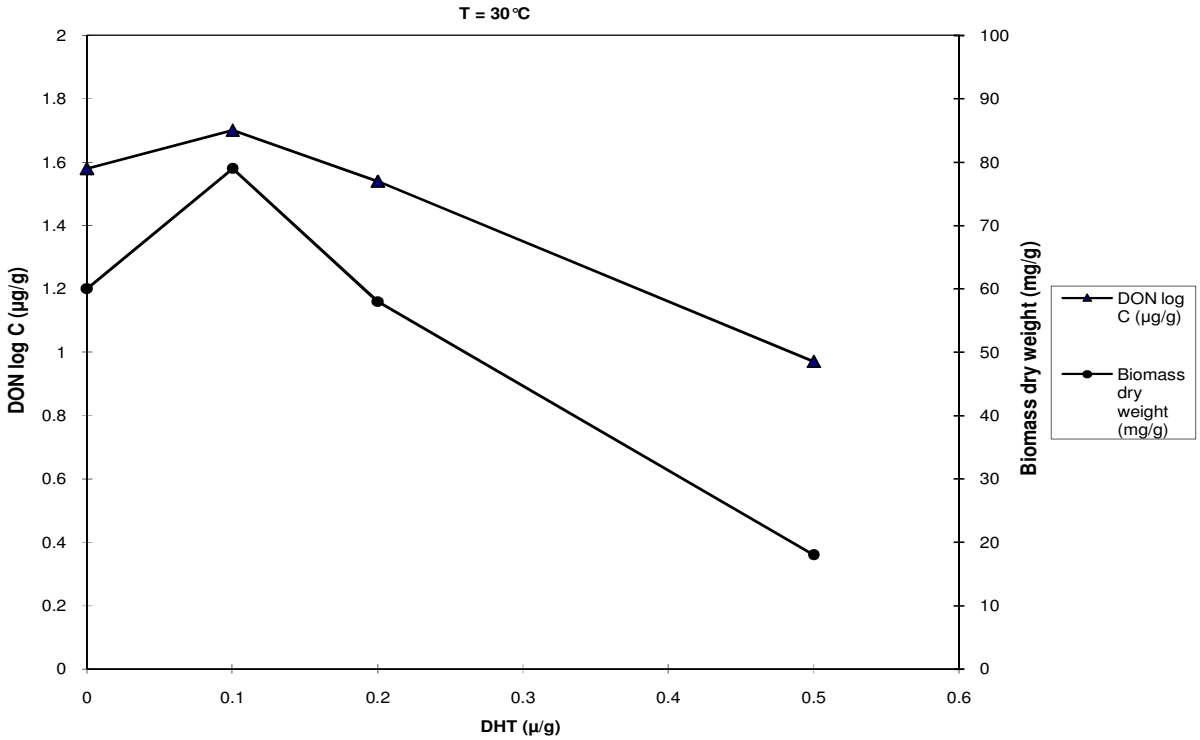

Figure 4. Comparative representation of biomass growth and DON accumulation during the cultivation of mould *F. graminearum* ZMPBF 1244 in the presence of different concentrations of DHT. Cultures were cultivated in maize grain at 30°C (measured after 35 days in relation to values obtained in the control).

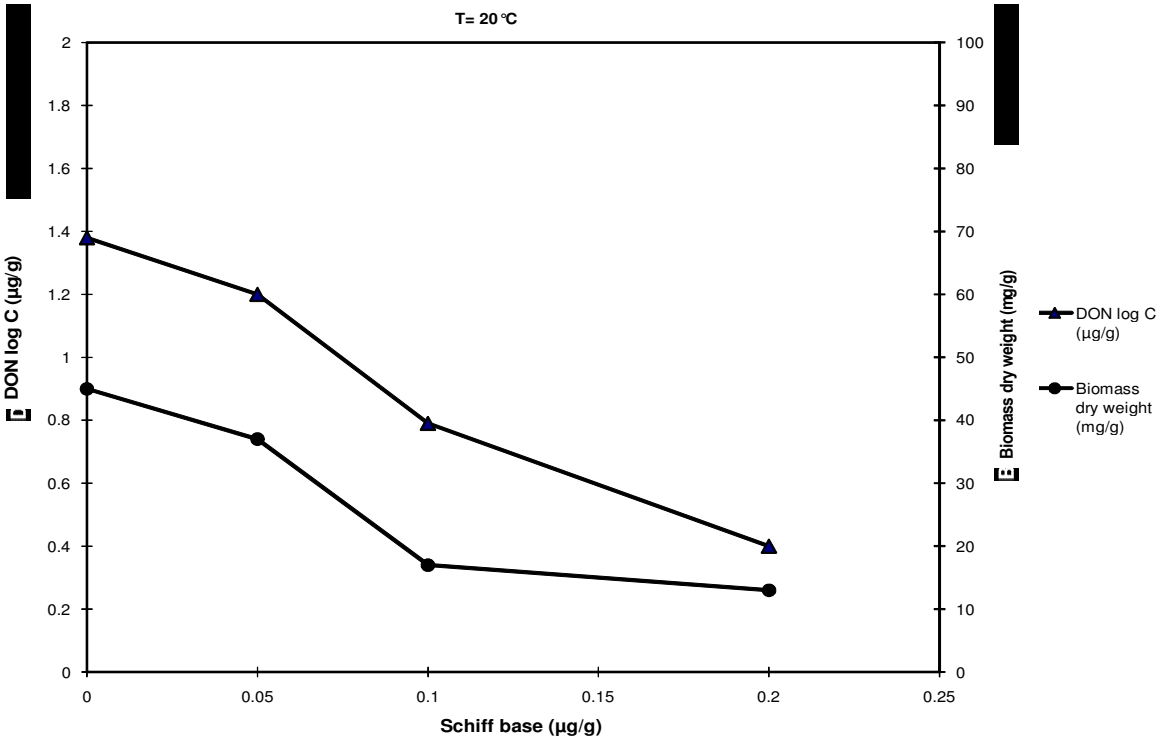

Figure 5. Comparative representation of biomass growth and DON accumulation during the cultivation of mould *F. graminearum* ZMPBF 1244 in the presence of different concentrations of Schiff base. Cultures were cultivated in maize grain at 20°C (measured after 35 days in relation to values obtained in the control).

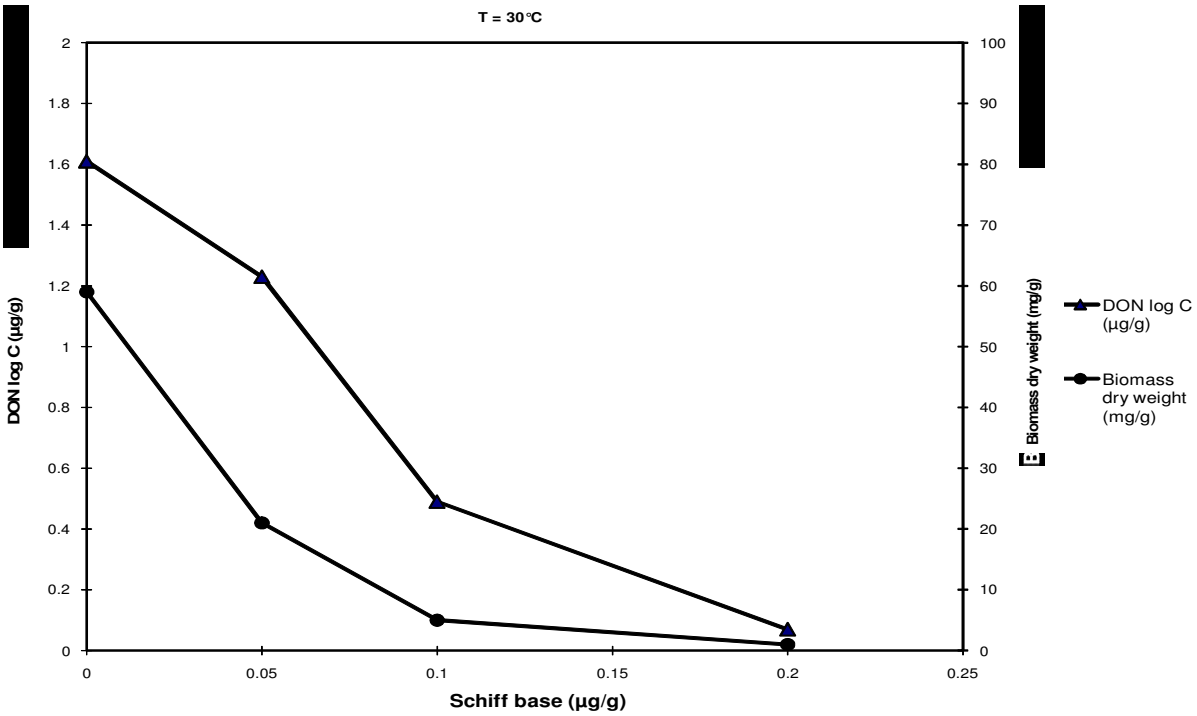

Figure 6. Comparative representation of biomass growth and DON accumulation during the cultivation of mould *F. graminearum* ZMPBF 1244 in the presence of different concentrations of Schiff base. Cultures were cultivated in maize grain at 30°C (measured after 35 days in relation to values obtained in the control).

Table 2. Effect of investigated DHT on growth and DON accumulation during the growth of mould *F. graminearum* ZMPBF 1244, measured after 35 days of cultivation on maize grain at 20°C.

DHT (µg/g)	20°C				
	Incubation time (days)	Biomass dry weight (mg/g)	DON (µg/g)	% Inhibition a_{100}	
				Growth	DON
0	7	27.8	b-	-	-
	21	47.5	15.8	-	-
	35	55.35	23.6	-	-
	49	59.4	12.7	-	-
0.1	7	43.1	b-	Mould growth and accumulation of DON were stimulated.	
	21	68.9	21.3		
	35	72.8	31.9		
	49	81.4	17.0		
0.2	7	26.3	b-	Mould growth and accumulation of DON are almost identically to control.	
	21	43.9	12.9		
	35	57.85	25.7		
	49	62.4	10.45		
0.5	7	6.3	b-	77.5	b-
	21	14.9	10.8	69.6	32.0
	35	13.85	15.9	75.0	30.0
	49	17.5	8.9	71.0	35.5

a_{100} = (test culture / control culture x 100); b- = not detectable DON; - = not detected.

Table 3. Effect of investigated DHT on growth and DON accumulation during the growth of mould *F. graminearum* ZMPBF 1244, measured after 35 days of cultivation on maize grain at 30°C.

DHT (µg/g)	30°C				
	Incubation time (days)	Biomass dry weight (mg/g)	DON (µg/g)	% Inhibition a_{100}	
				Growth	DON
0	7	40.5	b-	-	-
	21	50.8	26.2	-	-
	35	62.8	34.8	-	-
	49	68.6	18.7	-	-
0.1	7	56.7	b-	Mould growth and accumulation of DON were stimulated.	
	21	71.1	35.4		
	35	79.1	49.7		
	49	87.6	28.0		
0.2	7	42.4	b-	Mould growth and accumulation of DON were small stimulated.	
	21	53.2	27.9		
	35	58.8	33.85		
	49	64.95	21.7		
0.5	7	12.6	b-	69.0	b-
	21	19.4	3.2	62.0	88.0
	35	21.3	5.5	65.0	85.0
	49	18.5	3.15	73.0	83.0

a_{100} = (test culture/control culture x 100); b- = no detectable DON; - = not detected.

Table 4. Effect of investigated Schiff base on growth and DON accumulation during the growth of mould *F. graminearum* ZMPBF 1244, measured after 35 days of cultivation on maize grain at 20°C.

Schiff base (µg/g)	20°C				
	Incubation time (days)	Biomass dry weight (mg/g)	DON (µg/g)	% Inhibition a_{100}	
				Growth	DON
0	7	27.8	b-	-	-
	21	47.5	15.8	-	-
	35	55.35	23.6	-	-
	49	59.4	12.7	-	-
0.05	7	17.0	b-	39.0	b-
	21	28.9	8.15	41.0	48.5
	35	36.0	13.6	32.0	45.0
	49	39.6	6.95	34.0	45.0
0.1	7	10.8	b-	41.0	b-
	21	16.2	9.8	66.0	38.0
	35	18.4	6.5	60.0	55.0
	49	22.4	4.25	62.0	38.5
0.2	7	6.3	b-	77.0	b-
	21	10.5	b-	78.0	b-
	35	13.9	2.4	67.0	90.0
	49	12.7	0.65	79.0	95.0

a_{100} = (test culture/control culture x 100); b-= not detectable DON; - = not detected.

Table 5. Effect of investigated Schiff base on growth and DON accumulation during the growth of mould *F. graminearum* ZMPBF 1244, measured after 35 days of cultivation on maize grain at 30°C.

30°C					
Schiff base (µg/g)	**Incubation time (days)**	**Biomass dry weight (mg/g)**	**DON (µg/g)**	**% Inhibition a_{100}**	
				Growth	**DON**
0	7	40.5	b-	-	-
	21	50.8	26.2	-	-
	35	62.8	34.8	-	-
	49	68.6	18.7	-	-
0.05	7	15.2	b-	64.0	b-
	21	17.0	7.5	67.5	72.0
	35	21.3	12.4	65.0	67.0
	49	24.9	5.9	61.0	68.5
0.1	7	6.1	b-	85.0	b-
	21	6.75	2.85	87.0	89.0
	35	7.0	3.0	88.0	95.0
	49	9.9	1.7	85.0	91.0
0.2	7	c-	b-	c-	b-
	21	c-	b-	c-	b-
	35	c-	b-	c-	b-
	49	c-	b-	c-	b-

a_{100} = (test culture/control culture x 100); b- = not detectable DON; c- = no detectable growth;- = not detected.

antivomitoxin agent.

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Abbreviations: DHA, Dehydroacetic acid; **AFB_1,** aflatoxin B_1; **OTA,** ochratoxin A; **DON,** vomitoxin (deoxynivalenol); **Schiff base,** 3-/2-aminophenylamine-(p-toluoyl)-4-hydroxy-6-(p-tolyl)-2H-pyrane-2-one; **DHT,** 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one; **MIC,** minimal inhibitory concentration; **ZEA,** zearalenone; **PDA,** potato-dextrose agar; **TLC,** thin layer chromatography; **HPLC,** high performance liquid chromatography.

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