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GAS-CHROMATOGRAPHIC DETERMINATION OF TYPE A TRICHOTHECENE MYCOTOXINS IN EXTRACTS OF FUSARIUM SPP. ISOLATED FROM YUGOSLAV CORN HYBRIDS

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The study included 21 fungal cultures of Fusarium spp. isolated from Yugoslav corn kernels, as well as 3 control strains of F. sporotrichioides which are known producers of T-2 toxin and other type A trichothecenes. Liquid SYP medium, containing 5% sucrose 0.1% yeast extract and 0.1% peptone, was used for cultivation of fungi, for 5 days at 28^{0}C, on a rotatory shaker. Crude toxins were obtained by extraction with ethyl acetate. Purification was performed by application of SEP PAK C_{18} column chromatography and preparative thin-layer chromatography. The standard trichothecene mixture and purified extracts of fungal cultures were derivatised of with trifluoroacetic acid anhydride with addition of $NaHCO_3$. Trichothecene mycotoxin contents were determined by gaschromatography on an analytical capillary chromatographic column.

The presence of T-2 toxin was detected in 52% of the tested filtrates, of fungi which mainly weak producers of this trichothecene (0.5 - 15.0 mg/l). Besides T-2 toxin, DAS, T-2 tetraol and HT-2 toxin were detected only in extracts of the control F. sporotrichioides strains.

The methods for extraction, purification, derivatisation and determination of trichothecene mycotoxins by gas chromatography were modified allowing of very small amounts (0.5 ng for T-2 toxin, 0.2 ng for HT-2 toxin and DAS, and 0.1 ng for T-2 tetraol).

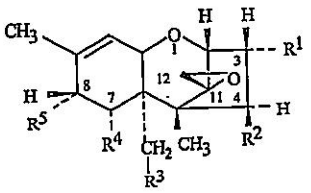
Key words: Corn, Fusarium spp, biosynthesis, type A trichothecenes, gaschromatographic determination.

INTRODUCTION

Among the epoxide fungal metabolites connected with human and animal intoxication, trichothecenes are the most important mycotoxins. These compounds are a chemically related group of biologically active secondary metabolites, produced by various species of the genera Fusarium, Myrothecium, Trichoderma, Cephalosporium, Stachybotrys, etc. (Ueno and Ueno, 1978. These trichothecene-producing fungi invade various agricultural products and a wide range of plants in field conditions as pathogens or parasites (Mc Gee, 1988).

Their biological characteristics govern the distribution and development of serious intoxications of man and animals, to a large extent. Fusarium spp. toxins classified as trichothecenes (T-2 toxin, deoxynivalenol=DON, diacetoxyscirpenol=DAS, nivalenol,, fusarenon X and 3-acetyl deoxynivalenol=3-ADON) singly or in combination, are thought to be responsible for numerous toxicoses (Bottalico et al. 1983; Watson et al. 1984) that are characterized by the following symptoms: stomatitis, dermatitis, haemorrhage, suppression of the haematopoietic system, depletion of the bone marrow with resultant leukopenia, thrombocytopenia and immunosuppression (Smalley and Strong, 1974). A considerable number of papers in Yugoslavia emphasize the continuous presence of type A trichothecene metabolites in samples of feedstuffs and fodder (Bočarov-Stančić and Protić, 1992; Lević et al. 1993). Within this group of mycotoxins, characterized by the presence of H or OH groups (free or esterified), located on the carbon atom in position 8 (Table 1), the greatest attention has been given to T-2 toxin, while little work has been done on the determination of its metabolite products (T-2 triol, HT-2) and DAS.

Table 1. Chemical structure of type A frichothecenes



	R	R	R	R	R
T-2 tetraol	OH	OH	OH	H	OH
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OA_c	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin	OH	OA_c	OA_c	H	$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpenol	OH	OA_c	OA_c	H	H

Numerous analytical methods are applied for the determination of trichothecene mycotoxins. Chromatographic methods utilized for the detection of trichothecene mycotoxins are thinlayer chromatogrophy (TLC), gas chromatography (GC) and high-pressure liquid chromatography (HPLC).

As trichothecenes do not contain chromophore groups in their structure, determination of their presence directly from TLC plates is insufficiently sensitive. Namely, the application of detection reagents is required for detecting trichothecenes by TLC chromatography. By spraying the chromatograms with

chromophoric reagents, spots are created which can be quantified densitometrically. The most widely applied chromophoric reagent for trichothecene mycotoxins is a 10-20% alcohol solution of sulfuric acid, while p-anisaldehyde, 4-p-nitrobenzylpyridine, Na-methoxide and cesium (IV) sulfate are less used. The detection limit for trichothecene mycotoxins after application of these reagents is approximately 100 ng per chromatographic spot (Ueno, 1983). Application of two-dimensional TLC improves the possibility for determination of trichothecenes and reduces the low detection limit for these mycotoxins. However, it enables the analysis of only one sample per chromatographic plate which consquently increases the cost and time required for performing this method.

The most suitable method for determination of polar, poorly evaporable organic compounds of high molecular weight, including trichothecenes, is HPLC. The absence of chromofores in trichothecenes leads to low molar absorption, thus reducing the plausibility of utilizing UV detectors in HPLC determination of these mycotoxins (Kuronen, 1989).

Trichothecenes are detected by GC only after derivatisation. Procedures include silanisation of these mycotoxins, to obtain methylsilanes, which are then traced by the flame-ionizing detector (FID). Derivatisation can also be performed by esterification of OH groups with anhydrides of fluorosubstituted organic acids, followed by electron capture detection (ECD) (Radošević, 1993; Croteau et al. 1994).

Other methods cited in the literature for analysis of trichothecene metabolites include nuclear magnetic resonance (NMR) spectroscopy, with application of ^{1}H and ^{13}C isotopes (Mesilaako et al., 1989) and radioimmunoassays (RIA) (Lee and Chu, 1981).

The objectives of this study were: 1) examination of Fusarium spp. isolated from Yugoslav corn kernels for T-2 toxin and other type A trichothecene production in laboratory conditions and 3) optimisation of a derivatisation method, and qualitative and quantitative GC determination of the mentioned mycotoxins.

MATERIALS AND METHODS

Fungal organisms. Different Fusarium species isolated from Yugoslav corn kernels harvested in 1990 (designated with M) and 1991 (designated with K, see Table 2), mainly originating from the Middle Banat region, were examined: F. graminearum (n=4), F. moniliforme (n=4), F. oxysporum (n=4), F. proliferatum (n=3), F. solani (n=2) and F. subglutinans (n=4). Strains of Fusarium sporotrichioides, known producers of T-2 toxin and other trichothecenes (KF-38/1 leg. J. Chelkowski, Poland; M-1-1 leg. Y. Ueno, Japan; R-2301 leg. E. Latus, Germany) were used as controls in this study. Stock cultures of fungi were maintained as suspensions in sterile water. Isolates of Fusarium spp., subcultured on potato-dextrose agar for 7 days at $28^{0}C$, were used as inocula for liquid medium.

Culture conditions. Erlenmeyer flasks (750 ml) containing 375 ml SYP medium (sucrose 50 g, yeast extract 1 g, peptone 1 g, distilled water up to

1l, pH-5.6) (Ueno et al. 1975) were autoclaved for 20 min at 120^{0}C. Media inoculated with spores of fungal isolates were incubated for 5 days at 28^{0}C on a rotatory shaker. All examinations were performed in duplicate.

A n a l y s i s. After filtration of liquid fungal cultures, the crude toxins were isolated from filtrates by extraction into ethyl acetate. The obtained extracts were evaporated to an oily residue on a rotatory vacuum evaporator and dissolved in a mixture of methanol +water (1 + 1 v/v). Purification of trichothecenes was performed by column chromatography according to the procedure described by Romer et al. 1978, and by application of SEP-PAK C_{18} columns for fast preparation of liquid extracts (Tokino and Yamaguchi, 1994). Following column chromatography, Fusarium extracts were subjected to preparative TLC on silica gel H (0.5 mm) plates for further purification. Purity of the obtained samples was tested by TLC on silica gel G (0.25 mm) plates and when satisfactory results were obtained, a GH analysis of trichothecene mycotoxins was performed. Derivatisation of trichothecenes was done by esterification of their alcohol groups with anhydrides of trifluoroacetic acid. Mycotoxin contents were determined with a "Varian" gas chromatograph type 3400. Trichothecenes were separated on a capillary analytical column 3% OV-101, 25 cm long, 0.32 mm inner diameter and 0.2 μm film size. The injector temperature was 230^{0}C and EC detector 290^{0}C. The column gas flow rate was 1.2 cm^3, with nitrogen "make up" of 25 cm^3/min.

RESULTS AND DISCUSSION

T o x i g e n i c i t y o f F u s a r i u m c u l t u r e s i s o l a t e d f r o m Y u g o s l a v c o r n. The results obtained for trichothecenes biosynthesized by Fusarium isolates from Yugoslav corn kernels, are presented in Table 2.

Current investigations are focused on Fusarium spp. isolated from corn grains, for corn represents one of the basic components of feed mixtures and is frequently used individually as a feedstuff for various species of farm animals. The predominant fungi found in corn grain harvested in 1990 and 1991 was F. subglutinans, followed by F. graminearum and F. moniliforme. These findings are similar to the results of Rheeder et al. 1995., obtained from commercial South African corn. With the exception of F. moniliforme M-26/2, isolated from white corn, all the other fungal cultures originated from different hybrids of yellow corn.

Out of a total of 21 tested Fusarium culture (Table 2), 52% were toxogenic i. e. biosynthesized T-2 toxin. None of the examined strains produced DAS or other type A trichothecenes, under the described laboratory conditions. The obtained results are in agreement with earlies studies on fungal isolates originating from Yugoslav corn (Bočarov-Stančić et al., 1986, Bočarov-Stančić et al., 1995.).

Within the Liseola section (including F. moniliforme, F. proliferatum, F. subglutinans), we detected the presence of T-2 toxin in filtrates of 5 out of 11 examined samples, in quantities of 0.5-3.0 mg/l (Table 2). Higher yields of this toxin were obtained from F. graminearum cultures (Discolor section) in amounts

Table 2. Biosynthesis of T-2 toxin by Fusarium cultures originating from corn harvested in 1990 (designated with M) and 1991 (designated with K)

No.	Species	Strain designation	T-2 toxin yield (mg/l)
1.	*Fusarium graminearum*	K-3/1	2.0
2.	*Fusarium graminearum*	K-14/B	0
3.	*Fusarium graminearum*	M-28/B	15.0
4.	*Fusarium graminearum*	M-GZ/P	0
5.	*Fusarium moniliforme*	K-12/1	0
6.	*Fusarium moniliforme*	M-3/2	0
7.	*Fusarium moniliforme*	M-26/2	0.5
8.	*Fusarium moniliforme*	M-27/1	0
9.	*Fusarium oxysporum*	K-3	0
10.	*Fusarium oxysporum*	K-12/2	1.0
11.	*Fusarium oxysporum*	K-13/4B	4.0
12.	*Fusarium oxysporum*	K-21/0	2.0
13.	*Fusarium proliferatum*	K-5/2	3.0
14.	*Fusarium proliferatum*	K-20/3P	0
15.	*Fusarium proliferatum*	K-21/AP	0.5
16.	*Fusarium solani*	K-4/1	0
17.	*Fusarium solani*	K-13	1.0
18.	*Fusarium subglutinans*	K-2/5S	0
19.	*Fusarium subglutinans*	K-6/1S	0
20.	*Fusarium subglutinans*	K-17/S	1.0
21.	*Fusarium subglutinans*	M-28/1S	1.0
22.	*Fusarium sporotrichioides*/cs	KF-38/1	150.0
23.	*Fusarium sporotrichioides*/cs	M-1-1	300.0
24.	*Fusarium sporotrichioides*/cs	R-2301	120.0

Legend : cs - control strain

of 2.0-15.0 mg/l. Three of four F. oxysporum strains, belonging to the Elegans section, biosynthesized T-2 toxin; but only in small amounts (up to 4.0 mg/l). Even lower yields were obtained from isolates of F. solani (Martiella section). According

to the literature available to us (Marasas et al., 1984; Lew, 1995), the majority of examined Fusarium species are potential producers of T-2 toxin and DAS, with the exception of F. proliferatum. In order to check the suitability of culture conditions for biosynthesis of T-2 toxin, a control strain of F. sporotrichioides R-2301 was simultaneously incubated. Table 2 shows that considerably higher yields of this mycotoxin were obtained in the latter case (120.0 mg/l).

The presented results show that Fusarium spp., isolated from Yugoslav corn in 1990 and 1991 did not have a high potential for biosynthesis of T-2 toxin. Since the majority of tested cultures was toxigenic, more attention should be given to trichothecene metabolites. Because trichothecenes are potential causative agents of a variety of mycotoxicoses in farm animals, the maximally permitted concentrations of these mycotoxins are consequently low, as stated in the current Book of Regulations printed in the Yugoslav Official Register (Sl. list SFRJ No. 2, 1990). These values must not exceed 0.30 mg/kg and 0.60 mg/kg in feedstuffs and feed mixtures, respectively.

Parameters for gas-chromatographic determination of type A trichothecenes. The following optimal conditions, for the determination of trichothecene derivates were obtained: a) the standard mixture of trichothecenes, dissolved in methanol was T-2 toxin 10.0 μg/ml, HT-2 toxin 2.5 μg/ml, T-2 tetraol 5.0 μg/ml and DAS 5.0 μg/ml; b) the acceptor of protons, formed after esterification of mycotoxin hydroxyl groups, was a dry powder of sodium-hydrogen carbonate causing increased sensitivity of the T-2 toxin detection method and slightly decreased sensitivity for its metabolites; c) the efficacy of esterification was highest during 30 min at 60°C; d) stability of newly-formed derivatives was longer than 5 days; e) the derivatisation procedure was performed in a special bottle with a teflon stopper; f) the obtained derivatives were extracted with a hexane+toluol (9+1, v/v) solvent system, containing a predetermined amount of internal standard the (organochloric insecticide metoxychlor); g) the hexane-toluol layer was directly injected into the GC.

In the stated conditions, a satisfactory separation of the standard trichothecene mixture was achieved (Table 3). In the investigated samples

Table 3. Retention times and relative retention times, compared to metoxychlor, for type A trichothecenes

No.	Substance	Retention time	Relative retention time
1.	T-2 tetraol	10.72	0.443
2.	DAS	11.80	0.488
3.	HT-2	21.17	0.875
4.	Metoxychlor	24.20	1.0
5.	T-2	33.85	1.399

obtained from Fusarium extracts isolated from corn, the yield of mycotoxins was considerably lower (up to 15.0 mg/l) in comparison to control F. sporotrichioides culture. (Table 2). Thus, T-2 toxin concentrations were significantly higher in the control cultures (KF-38/1 150.0 mg/l; M-1-1 300.0 mg/l; R-2301 120.0 mg/l) The applied procedures with the additional purification step utilizing column chromatography, using SEP-PAK columns filled with C_{18} phase and preparative TLC enabled the formation of extracts suitable for analysis on a highly sensitive EC detection limit for T-2 toxin was 0.5 ng for DAS and HT-2, and 0.1 ng for T-2 tetraol. If we assume that the analyzed sample weight does not exceed 50.0 g and the final volume is set at 0.2 ml before injection into the gas chromatograph, we may conclude that it is possible to quantitate T-2 toxin in concentrations lower than 2.0 μ g/kg. The detection limit for T-2 toxin metabolites is even lower, therefore detection in animal fluids is made possible.

The presented results are in agreement with those of Marasas et al. 1987, who analyzed 5 toxicologically important strains of Fusarium sporotrichioides. All fungal isolates were capable of biosynthesizing T-2 toxin, HT-2 toxin, HT-2, T-2 triol and neosolaniol, while T-2 tetraol and DAS were only sporadically detected. The sequence of appearance of these mycotoxins during GC detection was as follows: T-2 tetraol, DAS, neosolaniol, T-2 triol, HT-2 and finally T-2, which was the case in our study, also.

Recoveries of DAS and T-2 toxin averaged 80% and 85%, respectively in corn, and 70% and 90% in mixed feeds at concentrations ranging from 0.1 to 2.0 mg/kg. Recoveries of HT-2 and T-2 tetraol were 07% and 56%, respectively in corn at concentrations of 0.25 mg/kg (Rood et. al., 1988). The same authors estimated the detection limit to be 0.02 mg/kg in corn and mixed feeds by use of a capillary GC with an EC detector.

CONCLUSION

The presence of T-2 toxin was detected in 52% of the extracts of the tested Fusarium species. In laboratory conditions, the examined Fusarium isolates obtained from Yugoslav corn grains were mainly poor producers of T-2 toxin ranging from 0.5-15.0 mg/1. The presence of DAS, T-2 tetraol and HT-2 toxin was detected only in extracts of control F. sporotrichioides strains. Satisfactory sample purity was obtained by utilizing analytical procedures for extraction and purification of the primary trichothecene extracts, thus enabling their detection via the GC method. After derivatisation of trichothecene mycotoxins with anhydrides of trifluoroacetic acid, fluoroderivatives were obtained and quantitated on the EC detector in concentrations lower than one nanogram per microliter.

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GASNOHROMATOGRAFSKO ODREĐIVANJE TRIHOTECENSKIH MIKOTOKSINA TIPA A U EKSTRAKTIMA GLJIVIČNIH KULTURA

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SADRŽAJ

Ispitivanjem je bio obuhvaćen 21 izolat Fusarium spp. sa zrna kukuruza iz Jugoslavije, kao i 3 kontrolna soja F. sporotrichioides koji su poznati producenti T-2 toksina i drugih trihotecena tipa A. Kultivacija gljiva je izvršena u tečnoj SKP podlozi (5% saharoza + 0,1% ekstrakt kvasca + 0,1% pepton) tokom 5 dana na 28^{0}C. Sirovi toksini su dobijeni ekstrakcijom etil acetatom. Prečišćavanje je obavljeno kolonskom hromatografijom prema postupku Romer et al. (1978) ili primenom SEP-PAK C_{18} kolona, i preparativnom tankoslojnom hromatografijom na pločama presvučenim silika gelom H. Derivatizacija standardne smeše mikotoksina i prečišćenih ekstrakta gljivičnih kultura izvođena je anhidridom trifluorsirćetna kiseline uz dodatak $NaHCO_3$ kao akceptora protona. Određen je sadržaj trihotecenskih metabolita metodom gasne hromatografije na analitičkoj kapailarnoj hromatografskoj koloni.

U filtratima 52% testiranih gljivičnih kultura iz roda Fusarium je konstatovano prisustvo T-2 toksina. U pitanju su bili uglavnom slabi producenti ovog trihotecena (0,5-15,0 mg/l). DAS, T-2 tetraol i HT-2 toksin su utvrđeni samo u ekstraktima kontrolnih sojeva F. sporotrichioides.

Razvijen je postupak ekstrakcije, prečišćavanja, derivatizacije i kvantitativnog određivanja trihotecenskih mikotoksina tipa A koji je omogućio njihovu determinaciju apsolutnom unosu u instrument za gasnu hromatografiju od 0,5 ng za T-2 toksin, 0,2 ng za HT-2 toksin i DAS, i 0,1 ng za T-2 tetraol.