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INVESTIGATION OF TOXIGENIC POTENTIAL OF FUNGAL SPECIES BY THE USE OF SIMPLE SCREENING METHOD

ABSTRACT: Potential for the biosynthesis of aflatoxin B1 (AFLB1), ochratoxin A (OTA), diacetoxyscirpenol (DAS), T-2 toxin (T2), and zearalenone (ZON) was investigated in different fungal species belonging to the genera: *Aspergillus*, *Fusarium* and *Penicillium*. The majority of investigated isolates originated from cereal grains, crushed oil soybean seed and fodder mixtures. The simple screening method developed by Filtenborg et al. (1983) was applied with few modifications concerning the type of the medium and cultivation temperature. In order to optimise the biosynthetic conditions for different mycotoxins, the following control cultures, known as mycotin producers were used: OTA — *A. ochraceus* CBS 108.08, DAS — *F. semitectum* (SL-B i SL-C), T2 — *F. sporotrichioides* (ITM-391, M-1-1, R-2301) and ZON — *F. graminearum* (GZ-LES). The fungi were cultivated on the standard medium (YESA — 2% yeast extract, 15% sucrose and 2% agar, pH 6.5), three modifications of the basic medium ($YESA^{Zn}$ — the standard medium supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O; PPSA — the medium in which yeast extract was replaced with peptone-1; $PPSA^{Zn}$ — the medium in which yeast extract was replaced with peptone-1 and supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O), and the potato-dextrose agar (PDA).

The earlier biosynthesis of tested mycotoxins was recorded under the following cultivation conditions of fungal species: AFLB1 — after 14 days on PDA at 27±1°C, OTA — after 10 days on YESA and $YESA^{Zn}$ at 27±1°C, DAS — after 10 days on PPSA and $PPSA^{Zn}$ at 27±1°C, T2 — after 7 days on $PPSA^{Zn}$ and PPSA at room temperature (20—24°C), and ZON — after 1 week on YESA and $YESA^{Zn}$ at room temperature (21—24°C).

KEY WORDS: *Aspergillus*, *Fusarium, Penicillium*, screening, mycotoxins

INTRODUCTION

It is strongly recommended by many authors (Samson and van Reenen-Hoekstra, 1988) that dominant mycobiota of important food and feed in every country should be identified. Correct identification of dominant species can indicate which mycotoxin can be expected in a given food or feed item under given environmental conditions. In order to get the accurate data about mycotoxin profile of determined species, the simple screening method for toxigenic fungi was developed by few authors including Filtenborg et al. (1983).

In order to determine toxigenic profiles of fungal isolates from feed components and fodder mixtures in Serbia the present investigation was carried out. Considering the climatic conditions in our country, the first modification of the screening method of Filtenborg et al. (1983) was done in accordance with lower temperature for testing fusariotoxin production. Another modification of the same method was the addition of the trace element Zn in order to investigate its impact on the mycotoxin biosynthesis under tested laboratory conditions, and the third one was the replacement of yeast extract with peptone-1 in nutrient medium for screening fungal toxicity.

MATERIAL AND METHODS

Test microorganisms. Potential for the biosynthesis of aflatoxin B1 (AFLB1), ochratoxin A (OTA), diacetoxyscirpenol (DAS), T-2 toxin (T2), deoxynivalenol (DON) and zearalenone (ZON) was investigated in different fungal species belonging to the genera: *Aspergillus* (11), *Fusarium* (7) and *Penicillium* (3). Most investigated isolates, determined according to Samson and van Reenen-Hoekstra (1988), originated from cereal grains and fodder mixtures and were obtained during routine mycrobiological analyses in the Center for Bio-Ecology in 2008. Stock cultures of the fungi were maintained on the potato-dextrose agar at 4—6°C.

Control microorganisms:

— *Aspergillus ochraceus* **CBS 108.108**, the strain that produces OTA;

— *F. graminearum*, the isolate **GZ-LES** obtained in 1975 from corn kernels in Leskovac, that synthesises ZON and DON (Jajić et al., 2007);

— *Fusarium sporotrichioides* isolates, known as T2 toxin producers (Bočarov-Stančić et al., 2007): **ITM-391**, leg. dr A. Bottalico, Consiglio Nazionale delle Richerce, Istituto Tosssine e Micotossine da Parassiti Vegetali, Bari, Italy; **M-1-1** from soybeans, leg. dr Y. Ueno, Faculty of Pharmaceutical Sciences, Tokyo, Japan and **R-2301**, leg. dr D. Latus, Germany;

— *Fusarium semitectum* cultures **SL-B** and **SL-C** that biosynthesise DAS, isolated from unfaded alfalfa in 2004 (Bočarov-Stančić et al., 2005).

Stock cultures of the fungi were maintained on the potato-dextrose agar at 4—6°C.

Media and conditions for screening toxin production. The fungi were cultivated on the standard (Samson and van Reenen-Hoekstra,

1988) yeast extract-sucrose agar (YESA — 2% yeast extract, 15% sucrose and 2% agar, pH 6.5), with three modifications of that basic medium: (i) the yeast extract-sucrose agar supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O ($YESA^{Zn}$, pH 6.5) (Müchlencoert, 2004), (ii) the medium in which the yeast extract was replaced with peptone-1 (PPSA — 2% peptone-1, 5% sucrose and 2% agar, pH 6.5) and (iii) the medium in which yeast extract was replaced with peptone-1 and supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O ($PPSA^{Zn}$, pH 6.5), as well as on the potato-dextrose agar (PDA, pH 6.9).

In most cases, the temperature of 27±1°C was used for the fungal cultivation, although in the case of testing *Fusarium* isolates for fusariotoxins production, the cultivations were done parallely at room temperature. The cultivation period ranged from 7 to 14 days, depending on a type of mycotoxin and temperature conditions.

Determination of mycotoxins. Agar plugs were cut out of the colony center with sterile metal borer (diameter 6 mm), removed from the agar plate and placed with a sterile needle or tweezers in sterilized Petri dish with a mycelial side up. Circular plugs were wetted with 10—20 μL of chloroform/methanol (2:1 v/v) and after few seconds rapidly extracted mycelial side was gently applied against the TLC plate (MN Kieselgel G, Machery-Nagel) with sterile tweezers (Filtenborg et al., 1983). After drying the application spot, another one of the same colony was applied nearby together with 5 μl of the working standard of the tested mycotoxin (internal standard). The thin-layer chromatography was performed in saturated tanks with toluene/ethyl acetate/formic acid developing solvent (5:4:1, v/v/v) for all tested mycotoxins. Besides this solvent, only in the case of the ZON determination, benzene/glacial acetic acid developing solvent (90+10 v/v) was used. After developing plates and air drying in dark fume extractor the plates were examined under long wave UV light (366 nm). T2 and DAS were visualised by the use of 20% sulphuric acid in methanol, and ZON with 20% $AlCl_3$ in 60% ethanol. All analyses were done in three replicates.

RESULTS AND DISCUSSION

Results of the present investigation are shown in Tables 1—4.

Aflatoxin B1 (AFLB1). The ability to produce this mycotoxin was tested in 6 *Aspergillus* spp. isolates from crushed oil soybean seed and different types of fodder. Besides 3 *A. flavus* isolates (Table 1), one *A. clavatus* (T0001C/08), one *A. fumigatus* (RST-1/46), and one *A. versicolor* (RST-4/46) were also tested.

Tab. 1 — Production of aflatoxin B1 by *Aspergillus flavus* isolates

Sample designation	Temper. (°C)	Days	Medium				
			$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
GD-16/06	27±1	14	—	—	+	—	—
L09661/08	27±1	14	—	—	—	—	—
T0001F/08	27±1	14	—	—	—	—	—

Legend: — no biosyinthesis; + low intensity of biosynthesis

The obtained results show that only one *A. flavus* culture (GD-16/06) biosynthesised AFLB1 and only on PDA medium (Table 1). Other tested *Aspergillus* spp. isolates did not express potential for AFLB1 biosynthesis.

The cultivation time was exceeded to 14 days, because no traces of AFLB1 were observed after 10-day cultivation under the above conditions. In order to confirm the obtained results, another cultivation experiment was carried out with sterilised wet rice grain as a substrate for the toxin production. AFLB1 yield of 14 mg/kg of wet mass actually confirmed our results obtained by the use of modified method of Filtenborg et al. (1983), and showed once again that the optimal conditions for testing the AFLB1 production were: 27±1°C and 14-day cultivation on substrate containing starch.

Ochratoxin A (OTA). Screening of the OTA biosynthesis was done with 5 *Aspergillus* spp. and 3 *Penicillium* spp. isolates from crushed oil soybean seed, a complete mixture for calf growth and other feed types. Besides 3 *A. ochraceus* isolates (Table 2), one *A. alutaceus* (ZM/R), one *A. niger* (MA--K1/08), two *P. aurantiogriseum* (GD-8/06, MS/4a), and one *P. viridicatum* (OZK/R) were also tested. They all had the same toxigenic profile as *A. ochraceus* isolates — none of them produced OTA.

Tab. 2 — Production of ochratoxin A by *Aspergillus ochraceus* isolates

Sample designation	Temper. (°C)	Days	Medium				
			$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
GD-16/06	27±1	10	—	—	—	—	—
RST-2/46	27±1	10	—	—	—	—	—
T00010/08	27±1	10	—	—	—	—	—
CBS 108.08*	27±1	10	+	—	+++	++++	++++

Legend: * control strain, — no biosynthesis; + low intensity of biosynthesis, +++ high intensity, ++++ very high intensity

The *A. niger* isolate was included in our investigation because it was known from literature (Tjamos et al., 2004) that this species could produce various amounts of OTA in Corinth raisins and wine-producing vineyards in Greece. The only producer of this mycotoxin was a control strain *A. ochraceus* CBS 108.108 which optimal conditions for OTA biosynthesis were media with yeast extract and high sucrose content (YESA and $YESA^{Zn}$) (Table 2).

Fusariotoxins. Ability to produce ZON, DAS and T2 was investigated in 7 *Fusarium* isolates (*F. solani* — 1, *F. oxysporum* — 3, and *F. verticillioides* — 3) obtained from crushed oil soybean seed, different types of feed and soil for the indoor plant cultivation.

Tab. 3 — Production of zearalenone by *Fusarium* spp. isolates

Sample designation	Species	Temper. (°C)	Days	Medium				
				PPSA^{Zn}	PPSA	PDA	YESA^{Zn}	YESA
RST-3/46	*F. oxysporum*	21—24	7	—	—	—	—	+
			10	—	—	—	—	+
			14	—	—	—	—	+
MA-7A/08	*F. oxysporum*	21—24	7	—	—	—	—	—
			10	—	—	—	+	+
			14	—	—	—	++	++
MA-9/08	*F. oxysporum*	21—24	7	—	—	—	—	—
			10	—	—	—	—	+
			14	—	—	—	++	++
GZ-LES	*F. graminearum**	21—24	7	—	+	—	+	++
			14	—	+	—	+++	++

Legend: * control strain, — no biosynthesis; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity

Zearalenone (ZON) biosynthesis was detected only by *F. oxysporum* isolates and the control *F. graminearum* culture GZ-LES. At room temperature varying from 21 to 24°C, earlier formation of ZON was detected in some isolates after 7 days of cultivation, or after 10 days in others on YESA (Table 3), although higher quantities of the same fusariotoxin were detected after a prolonged cultivation (14 days). Other authors also reported the formation of the maximum amount of ZON after 12 days at 20°C by *F. oxysporum* and other *Fusarium* species isolated from cereal grains (El-Kady and El-Maragh y, 1982). In the control strain GZ-LES some smaller quantities of ZON were observed in the PPSA medium in which the yeast extract was replaced with the same concentration of peptone-1.

Diacetoxyscirpenole (DAS). None of the tested isolates of *Fusarium* spp. biosynthesised this mycotoxin of type A trichothecene. Results obtained with 2 control strains of *F. semitectum* (Table 4) point out that the best medium for screening the ability to produce DAS under laboratory conditions was PPSA in which the yeast extract was replaced with the same concentration of peptone-1 (2%). These results are, to a certain degree, surprising because our previous investigations (Bočarov-Stančić et al., 2003) showed that the isolate SL-B could produce DAS in the liquid media with yeast extract and peptone-1. We assume that the cultivation temperature (27±1°C), higher than in previous investigations, is the reason for such a result.

Tab. 4 — Production of trichothecenes of type A by *Fusarium* spp. control isolates

Species	Sample design.	Temp. (°C)	Days	Medium				
				$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
Diacetoxyscirpenol								
F. semitectum	SL-B	27±1	10	++	+++	—	—	—
F. semitectum	SL-C	27±1	10	+	++	—	—	—
T-2 toxin								
F. sporotrichioides	ITM-391	20—24	7	+++	++	n.a.	++	++
			10	+++	++++	n.a.	+++	++++
		27±1	10	++	+++	+	n.a.	n.a.
F. sporotrichioides	M-1-1	20—24	7	++	+	n.a.	+	+
			10	++	+++	n.a.	++	++
		27±1	10	++	++	+	n.a.	n.a.
F. sporotrichioides	R-2301	20—24	7	++++	+++	n.a.	+++	+++
			10	++++	++++	n.a.	++++	++++
		27±1	10	+++	++++	n.a.	n.a.	n.a

Legend: n.a. — not analysed; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity, ++++ very high intensity

T2 toxin. As in case of DAS, none of the tested isolates of *Fusarium* spp. biosynthesised this another mycotoxin of type A trichothecene (Table 4), although according to Marasas et al. (1984), our previous investigations (Mašić et al., 1997), and Sokolović et al. (2008) some isolates belonging to the species *F. solani*, *F. oxysporum* and *F. verticillioides* can produce T2.

In the case of control *F. sporotrichioides* isolates, the earliest T2 biosynthesis was observed after 7 days of cultivation on all media with 15% of sucrose, although the highest yields of this fusariotoxin were observed after a prolonged cultivation (10 days) at room temperature (20—24°C). The medium PDA was unsuitable for screening the ability to produce T2 (Table 4). On the other hand, the best results were obtained after cultivation on PPSA or YESA, i.e. media with high sucrose concentration, regardless of the use of peptone-1, or the yeast extract as an N atom source. The addition of a small Zn concentration (0.23 mg/l $ZnSO_4$ x 5 H_2O) did not affect the toxin production although some authors point out that trace microelements can significantly influence the mycotoxin production (Mühlencoert, 2004).

CONCLUSIONS

For screening ability to produce different mycotoxins, the optimal cultivation conditions are as follows:

— AFLB1 — 14 days on PDA and 27±1°C;

— OTA — 10 days on YESA and $YESA^{Zn}$ at 27±1°C;

— ZON — 2 weeks on YESA and $YESA^{Zn}$ at room temperature (21—24°C);

— DAS — 10 days on PPSA and $PPSA^{Zn}$ at 27±1°C;

— T2 — 7 days on $PPSA^{Zn}$ and PPSA at room temperature (20—24°C).

Trace element Zn (0.23 mg/l $ZnSO_4$ x 5 H_2O) did not affect the mytoxin production in tested conditions.

Analysed fungal isolates from cereals and fodder in Serbia were mainly non toxic — out of 21 only one culture of *A. flavus* produced AFB1, and three *F. oxysporum* cultures produced ZON.

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ИСПИТИВАЊЕ ТОКСИГЕНОГ ПОТЕНЦИЈАЛА ГЉИВА ПРИМЕНОМ ЈЕДНОСТАВНОГ ТРИЈАЖНОГ МЕТОДА

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Резиме

Потенцијал за биосинтезу афлатоксина Б1 (АФЛБ1), охратоксина А (ОТА), диацетоксисцирпенола (ДАС), Т-2 токсина (Т2) и зеараленона је испитан код изолата гљива које припадају родовима *Aspergillus*, *Fusarium* и *Penicillium*. Изолати су углавном били пореклом са зрна житарица и сточне хране. Примењена је једноставна тријажна метода Filtenborg-a и сар. (1983) код које су извршене извесне модификације у смислу типа подлоге и температуре култивације гљива.

У циљу оптимизације услова за тестирање токсигеног профила одабраних гљивичних изолата употребљене су контролне културе за које је претходно доказано да су произвођачи следећих микотоксина: ОТА-а *A. ochraceus* CBS 108.08, ДАС-а *F. semitectum* (СЛ-Б и СЛ-Ц), Т2 — *F. sporotrichioides* (ITM-391, M-1-1, R-2301) и ZON-а *F. graminearum* (ГЗ-ЛЕС). Гљиве су гајене на стандардној подлози (ЕКСА — 2% екстракта квасца и 15% сахарозе, pH 6,5), три модификације основне подлоге (EKSA^{Zn-} — стандардна подлога са додатком 0,23 mg/l $ZnSO_4$ x 5 H_2O; ППСА — подлога у којој је екстракт квасца замењен пептоном-1; ППСА^{Zn} — подлога у којој је екстракт квасца замењен пептоном-1 и којој је додато 0,23 mg/l $ZnSO_4$ x 5 H_2O; pH 6,5) и кромпир декстрозној подлози (pH 6,5).

Биосинтеза испитаних микотоксина је најраније констатована при следећим условима гајења гљива: АФЛБ1 — после 14 дана култивације на КДА и 27±1°C, ОТА — после 10 дана култивације на ЕКСА и EKSA^{Zn} и 27±1°C, ДАС-а после 10 дана култивације на ППСА и ППСА^{Zn} на 27±1°C, Т2 — после 7 дана на ППСА и ППСА^{Zn} и собној температури (20—24°C), и ZON-а после недељу дана на ЕКСА и ЕКСА^{Zn} и собној температури (21—24°C).