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OPTIMIZATION OF LABORATORY CONDITIONS FOR BYOSINTHESIS OF TYPE A TRICHOTHECENES

ABSTRACT: Type A trichothecenes, T-2 toxin and diacetoxyscirpenol — DAS, belong to one of the most toxic groups of fusariotoxins. Although larger quantities of them can be found more often in cooler parts of Europe, regarding their methabolic characteristics and the types of illnesses they provoke, it is obvious that even smaller quantities of these toxins can cause serious health disturbances of humans and animals in climatic conditions of Serbia.

Having in mind the importance of these substances, the aim of this study was to carry out the optimization of laboratory conditions under which screening of *Fusarium* spp. isolates from Serbia, regarding T-2 toxin and DAS production, should be done.

Four cultures of *Fusarium sporotrichioides*, originating from different regions throughout the world, were under present investigation: ITM-391 (Italy), KF-38/1 (Poland), M-1-1 (Japan) and R-2301 (Germany). According to the previous literature data, all of these isolates were T-2 toxin producers, and some of them were also DAS producers. The influence of medium composition (different C and N atoms sources, microelements etc.), as well as aeration (in liquid media), on byosinthesis process of these mycotoxins, *in vitro* conditions was investigated. In the case of most *Fusarium sporotrichioides* isolates, highest yields of T-2 toxin and DAS were achieved under the conditions of more intense aeration, and with the use of glucose (5 or 20%) as a C atom source. Fermentation in semi-synthetic liquid medium, using a rotary sheaker, was more suitable for screening the toxicity of the fungal isolates in pure culture because of: shorter period of incubation, more simpler sample preparation, obtaining less interfering materials in crude toxin extracts, and possibility for more precise definition of factors influencing the yield of trichothecenes.

KEY WORDS: biosynthesis, DAS, *Fusarium sporotrichioides*, T-2 toxin

INTRODUCTION

Representatives of fungal genus *Fusarium* are primarily plant pathogens, and occur mostly in association with plants and cultivated soils. Unlike most *Aspergillus* and *Penicillium* species, fusaria grow on crops before harvest, and only at high water activity (aw) levels in the substrate. Therefore, their toxic metabolites are produced before, or immediately after the harvest. Kimura et al. (2001) demonstrated that there is a causal role of fusaria mycotoxins in plant disease development. In case of some host-pathogen interactions, it was proved, by the use of contemporary molecular genetic techniques, that fusariotoxins are highly associated with disease initiation or enhanced virulence.

Several *Fusarium* species present on cereals worldwide, causing "head blight" (scrab) of small grain cereals, or "ear rot" of corn, are capable to accumulate, in infested kernels, few mycotoxins, some of which have the relevant impact on human and animal health. One of the main groups of *Fusarium* toxins are trichothecenes, with T-2 toxin, HT-2 toxin, neosolaniol, fusarenon-X etc. (Marasas et al., 1984). The primary mechanism of action of these strongly toxic sesquiterpenes, with tricyclic nucleus and an epoxide at C-12 and C-13, is the inhibition of protein synthesis at the level of a ribosome. 60S-ribosomal protein L3 is the major site of this inhibitory activity (Desjardins, 2003). The main simptoms of illnesses caused by these compounds, are vomiting, inflammation, diarrhoea, cellular damage of the bone marrow, thymus, spleen and mucous membrane of the intestines, and depression of circulating white blood cells (Marasas et al., 1984).

The presence of type A trichothecenes (T-2 toxin and diacetoxyscirpenole — DAS) in central and northeastern Europe is conecteded with *Fusarium* species *F. poae* and *F. sporotrichioides* (Bottalico, 1998). In mid-70s of the last decade proved that these fungi and their trichotecenes were associated with the death of at least 100,000 Russian people in the period 1942—1948. This exceptionally unpleasant disease, now called Alimentary Toxic Aleukia (ATA), is characterized by fever, haemorraghic rash, necrotic angina, extreme leucopenia, agranulocytosis, sepsis and exhaustion of bone marrow (Joffe, 1978). These symptoms resemble more closely those of radiation sickness, than bacterial or fungal toxicoses.

Besides T-2 toxin, *F. sporotrichioides* can biosynthesize other mycotoxins too, of which the most important are DAS, butenolide, fusarenon-X, neosolaniol and nivalenol primarily (Marasas et al., 1984). Although DAS is less toxic than T-2 toxin, in general it shows similar effects on animals and humans. Fortunately, *F. sporotrichioides* is not so commonly occurring species. It can be found mainly in temperate regions with cereals as main crops, although it has been also isolated from peanuts and soybean (Pitt and Hocking, 1985).

The production of trichothecenes proceeds from farnesyl pyrophosphate via hydrocarbon trichodiene. A sequence of oxygenations, isomerisations, cyclizations, and esterifications leads from trichodiene to more complex trichothecenes such as T-2 toxin, DON and NIV. Until now, eleven genes (*TRI3* — *TRI13*) coding trichothecene biosynthesis have been located, and their function

has been established by the target gene disruption in *F. graminearum* and *F. sporotrichioides* (D e s j a r d i n s, 2003).

Y u (2001) tried to give the answer on the following questions: how biosynthesis of various mycotoxins is controlled, and whether there are global controlling mechanisms for both sporulation and mycotoxin production. Investigations of this author revealed the existence of correlation with the pivotal role of signal transduction in cellular regulatory, communicatory, and responsive process, that led him to the hypothesis that heterometric G-protein signalling components and RGS proteins are upstream determinants in controlling processes of growth and development of *F. sporotrichioides.* From three *Fusarium* species (*F. graminearum, F. sporotrichioides* and *F. verticillioides*) important RGS protein was identified, as well as EST sequences that identify 9 out of 15 target signaling components in *F. sporotrichioides.*

Having in mind the importance of type A trichothecenes and toxin-producing *Fusarium* species, the aim of this study was to carry out the optimization of laboratory conditions for toxin biosynthesis of type A trichochecenes (T-2 toxin and DAS) in pure culture, in order to obtain faster and simpler procedure for screening of toxigenic potential of *Fusarium* spp. isolates.

MATERIAL AND METHODS

Microorganisms. *F. sporotrichioides* isolates, known as T-2 toxin producers, and some of them as DAS producers too, were under present investigation: 1) ITM-391, leg. dr A. Bottalico, Consiglio Nazionale delle Richerce, Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; 2) KF-38/1 from barley, leg. dr Y. Chelkowski, Department of Plant Pathology, The Agricultural Faculty, Warsaw, Poland; 3) M-1-1 from soybeans, leg. dr Y. Ueno, Faculty of Pharmaceutical Sciences, Tokyo, Japan and 4) R-2301, leg. dr D. Latus, Germany (B o č a r o v - S t a n č i ć and M u n t a ñ o l a - C v e t k o v i ć, 1988; N a g a y a m a et al., 1988; M a š i ć et al., 1997). Stock cultures of the fungi were maintained on potato-dextrose agar at 4—6°C.

Inoculation of fermentation media was performed with 5 pieces (5 x 5 mm) of fungal material originated from Petri dish, sowed with tested culture, and subcultivated for 7 days on potato-dextrose agar (PDA) at 27°C.

Cultivation conditions. 1) Inoculated Erlenmayer flasks (500 ml), containing 100 or 250 ml of different semisynthetic liquid media, were cultivated on rotary sheaker (150 or 180 rpm) at room temperature (21—26°C); 2) inoculated Roux bottles, containing 50 g of wet sterilized corn kernels (initial moisture 58.4%), were cultivated at 30°C for 4 weaks. All cultivations were performed in duplicate.

Liquid fermentation media: 1) ***GPY*** (5% of glucose + 0.1% of peptone + 0.1% of yeast extract) pH 5,8; 2) ***GPY^{Zn}*** (5% of glucose + 0.1% of peptone + 0.1% of yeast extract + 0.0009% of $ZnSO_4$ x 7 H_2O) pH 5,9; 3) ***SPY*** (5% of saccharose + 0.1% of peptone + 0.1% of yeast extract) pH 6,5 and 4) ***GPYM*** (20% of glucose + 0.4% of peptone + 0.1% of yeast extract + 0.05% of

K_2HPO_4 x $3H_2O$ + 0.025% of $MgSO_4$ x 7 H_2O + 0.025% of KCl + 0.0009% of $ZnSO_4$ x 7 H_2O) pH 5,8.

Analysis of fusariotoxins: 1) After cultivation on rotary sheaker, liquid cultures were filtered. Crude extracts of type A trichothecenes (DAS i T-2 toxin) were obtained by the use of ethyl acetate. Further purification was done by the method of Romer et al. (1978), while thin layer chromatography was performed according to Pepeljnjak and Babić (1991). 2) The samples obtained after cultivation on corn grain, were dried during 24 h or more, at 60°C, until constant weight was obtained. After pulverization of dried samples, type A trichothecenes were extracted and purified from them, by the method of Romer et al. (1978). Thin layer chromatography was done in the same way as previously.

RESULTS AND DISCUSSION

During present investigations in liquid semi synthetic media, the influence of aeration, C atom source and concentration, as well as mineral supplements on *F. sporotrichioides* toxigenicity *in vitro* conditions was investigated (Table 1). In the case of temperature conditions, cultivation of fusaria was not performed when temperature stress conditions, according to Joffe (1983), but at relatively constant room temperature (21—26°C), having in mind the observation of Richardson et al. (1985), and the one of Rabie et al. (1986), what in warmer parts of the world, such as North Carolina or South Africa, toxigenic strains of *Fusarium* spp., associated with fusatiotoxicoses of domestic animals, can be found.

Toxigenic potential of *F. sporotrichioides* isolates. Having in mind the results of our previous investigations (Bočarov-Stančić et al., 1986; Muntañola-Cvetković et al., 1991), which proved that *F. sporotrichioides* cultures originated from Serbian cereals had toxigenic potential, this species was chosen for optimisation of laboratory conditions for type A trichothecene production.

All tested *F. sporotrichioides* cultures biosynthesized T-2 toxin independently of cultivation conditions, although the best results were achieved in liquid glucose media (***GPYM*** and ***GPY***). The best producer of this type A trichothecene was ITM-391 isolate, in which yields ranging from 0.16—120.0 mg/l were recorded (Table 1). Lower concentrations of T-2 toxin obtained in the present investigation from strain: KF-38/1, M-1-1 and R-2301, than those obtained in the investigation of Mašić et al. (1997), can be explained by the fact that under laboratory conditions, important deteriation of toxigenic potential can be observed regardless of their preservation conditions (Bočarov-Stančić et al., 1989). Although in some cases, as proved by Joffe and Yagen (1977), isolates can even after 30 years of cultivation on artificial media, retain the original high biosynthetic capacity, if isolations of strains were done in other ecological conditions.

DAS was recorded in 75% of tested isolates, but exclusively in higher aeration conditions (180 rpm) in liquid media with ***GPYM*** and/or ***GPY***. The best

producer of this fusariotoxin was the isolate from Polish barley KF-38/1, which biosynthesized from 1.6—12.0 mg/l. During the cultivation of the same culture on moist, sterile natural substrate (Table 1), not only that duration of the cultivation was much longer (28 days in comparison to 3 days for submerged cultivation), but also purification procedure was more complicated, because of the presence of more interfering substances. Besides that, much lower yields of T-2 toxin and DAS were obtained (Table 1). Although other authors, such as Pereira and Kemmelmeier (2000), use similar cultivation conditions for investigation of toxigenicity of fusaria (natural substrate, 21 days at 25°C), better results can be achieved in liquid media because of more precise definition of the factors influencing trichothecene production, and obtaining cleaner extracts, in which simpler purification is necessary, so that loss of toxin is less outstanding. The results of the present investigation confirm our previous conclusions (Bočarov-Stančić and Muntañola-Cvetković, 1988).

The obtained results point out the weak toxigenic potential for DAS production, but not inadequate cultivation conditions in liquid semi-synthetic media, because high yields of the same fusariotoxin (64.0 mg/l) can be achieved by other fusaria, such as *F. semitectum* under the similar cultivation conditions (Bočarov-Stančić et al., 2005).

Aeration. During submerged cultivation on rotary sheaker with higher aeration (180 rpm), the highest yields of type A trichothecenes were achieved in ***GPY*** enriched with mineral supplements (***GPYM***), and with smaller volume of liquid medium in cultivation flasks (100/500 ml). According to data presented in Table 1. yields of T-2 toxin ranged from 12.0 to 120.0 mg/l, and DAS from quantities below detection limits up to 12.0 mg/l, respectively. Under the same conditions, a little bit weaker, but rather high yields were of type A trichothecenes, obtained in ***GPY*** (250/500 ml): T-2 toxin from 8.0 to 120.0 mg/l, and DAS from quantities below detection limits up to 8.0 mg/l. Although the same concentration of microelement Zn was present in ***GPYM*** and ***GPY^{Zn}***, poorer results were obtained: T-2 toxin from 0.08—0.16 mg/l (values near detection limits of applied TLC method), while DAS was not detected under the same conditions (Table 1).

In the case of submerged cultivation with lower aeration (150 rpm), the best results were achieved in ***GPY*** medium (250/500 ml). Although DAS was not detected in this case, the yields of T-2 toxin ranged from 8.0 to 64.0 mg/l. Data shown in Table 1 point out that higher aeration had positive effect on type A trichothecene biosynthesis, although the influence of media composition could not be neglected.

C atom source. The best results were achieved in ***GPYM*** medium (T-2 toxin from 12.0—120.0 mg/l, and DAS max. 12.0 mg/l, respectively). In the case of the same media composition, except that different sugars were used as C atom sources, the obtained results are presented in Table 1. ***GPY*** medium resulted in higher yields of T-2, in 75% of tested samples, and DAS in 100% of the samples during cultivation under higher aeration conditions (180 rpm). In the case of ***SPY*** medium, concentration of DAS was below detection limit,

Tab. 1. — Yield of type A trichothecenes (DAS, T-2 toxin) under different cultivation conditions of *F. sporotrichioides*

Isolate design.	Cult. condit. / Fusariot.	Fusariotoxin yield (mg/l or mg/kg)									
		250/500 ml						100/500 ml			
		GPY		*GPY^{Zn}*	*SPY*		*GPYM*	*GPY*	*SPY*	Corn grain	RANGE (mg/l or mg/kg)
		180 rpm	***150 rpm***	**180 rpm**	**180 rpm**	***150 rpm***	**180 rpm**	**180 rpm**	**180 rpm**		
ITM-391	DAS	3.2	*n.d.*	n.d.	n.d.	*n.d.*	4.0	—	—	—	**n.d. — 4.0**
	T-2	120.0	*64.0*	0.16	48.0	*64.0*	120.0	—	—	—	**0.16—120.0**
KF-38/1	DAS	8.0	*n.d.*	n.d.	n.d.	*n.d.*	12.0	0.24	n.d.	1.6	**n.d. — 12.0**
	T-2	64.0	*32.0*	0.08	2.4	*0.32*	20.0	4.0	0.16	2.4	**0.08—64.0**
M-1-1	DAS	n.d.	*n.d.*	n.d.	n.d.	*n.d.*	12.0	—	—	—	**n.d. — 12.0**
	T-2	8.0	*12.8*	0.08	16.0	*0.8*	80.0	—	—	—	**0.8—80.0**
R-2301	DAS	n.d.	*n.d.*	n.d.	n.d.	*n.d.*	n.d.	—	—	—	**n.d.**
	T-2	24.0	*8.0*	0.16	1.6	*1.92*	100.0	—	—	—	**0.16—100.0**
RANGE (mg/l or mg/kg)	**DAS**	**n.d. — 8.0**	***n.d.***	**n.d.**	**n.d.**	***n.d.***	**n.d. — 12.0**	—	—	—	
	T-2	**8.0—120**	***8.0—64.0***	**0.08—0.16**	**1.6—48.0**	***0.32—64.0***	**12.0—120**	—	—	—	

Legend: n.d. — not detected (< 0.08 mg/l or mg/kg)

while in the case of T-2 toxin, 50% of samples, tested under conditions of lower aeration (150 rpm), yielded higher quantities of the same mycotoxin (0.32—64.0 mg/l).

Results presented in Table 1 show that in the case of same applied concentration of C atom source, glucose had more positive effect on type A trichothecene biosynthesis than succrose, although other authors (Ueno et al., 1975) did not observe the essential influence of sugar type on T-2 toxin and fusarenon-X production.

N atom source and mineral supplements. On the basis of literature data it can be hardly concluded how large is the influence of N atom source, as well as mineral and vitamin supplements on *Fusarium* fermentation process under laboratory conditions. According to Cullen et al. (1982) highest yields of T-2 toxin can be achieved during cultivation in Vogels' mineral medium with 5% of glucose after 12—14 days at 15°C. Contrary to this finding, Muntañola-Cvetković et al. (1991) did not observe the positive influence of microelements and vitamin supplements on T-2 toxin yield, according to Vogel (1956). We had similar doubts about the influence of these paramethers while analyzing present results (Table 1). In the case of addition of Zn (0.00009%) to basic ***GPY*** medium, in order to accelerate the sporulation, as well as DAS and T-2 toxin biosynthesis, the outstanding decrease of toxin yields was observed. Contrary to that observation, in ***GPYM*** medium (same Zn concentration, but higher content of peptone — 0.04%, and sugar — 20.0%, as well as presence of different mineral supplements) the best toxigenic results were obtained (Table 1).

CONCLUSIONS

All tested cultures of *F. sporotrichoides* were better T-2 toxin producers (max. 120.0 mg/l) than DAS producers (max. 12.0 mg/l).

Highest yields of both type A trichothecenes were obtained under higher aeration conditions (180 rpm), as well as during the fermentation of smaller media volume in cultivation flask (100/500 ml in comparison to 250/500 ml).

Glucose, in quantities ranging from 20% (***GPYM***) under 5% (***GPY***) in given cultivation conditions i.e. independently of applied aeration, influenced more favorably T-2 toxin, and DAS production than succrose did.

The addition of microelement Zn in standard glucose medium (***GPY***), with the aim to obtain better sporulation and biosynthesis of fusariotoxins, did not give the expected results. In the last case, very low quantities of T-2 toxin were detected, near the detection limits of applied TLC method.

It was demonstrated that submerged cultivation in nutrient media with glucose can be the most suitable, cheapest and most rapid method for large scale screening of *Fusarium* isolates regarding the trichothecene production.

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ОПТИМИЗАЦИЈА УСЛОВА ЗА БИОСИНТЕЗУ ТРИХОТЕЦЕНА ТИПА А У ЛАБОРАТОРИЈСКИМ УСЛОВИМА

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Резиме

Трихотецени групе А, Т-2 токсин и диацетоксисцирпенол — ДАС, представљају једну од најтоксичнијих група фузариотоксина. Они се у већим концентрацијама чешће могу наћи у хладнијим регионима Европе, али, у складу са њиховим карактеристикама и врстама обољења која изазивају, јасно је да и њихове мање количине могу довести до озбиљних здравствених поремећаја код људи и животиња у климатским условима Србије.

С обзиром на значај ових једињења, циљ овог истраживања је био да се изврши оптимизација лабораторијских услова у којима би се испитивала способност за биосинтезу Т-2 токсина и ДАС-а код *Fusarium* изолата из Србије.

Истраживањем су биле обухваћене 4 културе *F. sporotrichioides* пореклом из различитих земаља света: ИТМ-391 (Италија), КФ-38/1 (Пољска), М-1-1 (Јапан) и Р-2301 (Немачка), за које је претходно описано у литератури да су продуценти Т-2 токсина, а неке и ДАС-а. Испитан је утицај састава подлоге (различити извори атома угљеника и азота, микроелементи и сл.) као и аерације (у случају течних подлога) на процес биосинтезе ових микотоксина *in vitro* условима. Код већине изолата највећи приноси Т-2 токсина и ДАС-а су добијени у условима веће аерације и при употреби глукозе (5 или 20%) као извора угљениковог атома. Ферментација у течној подлози се показала као погоднија метода за тестирање токсигености гљивичних изолата од природног стерилног супстрата, због краћег периода култивације, добијања сирових екстраката токсина са мање пратећих материја, као и могућности прецизнијег дефинисања фактора који утичу на принос трихотецена.