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IN VITRO DEGRADATION OF DIACETOXYSCIRPENOL AND T-2 TOXIN BY USE OF *MUCOR RACEMOSUS* FRESEN. *F. RACEMOSUS* ISOLATE*

ABSTRACT. Under controlled *in vitro* conditions the capacity of the *Mucor racemosus* f. *racemosus* 1215/09 isolate to degrade type A trichothecenes (diacetoxyscirpenol – DAS and T-2 toxin) was observed in the liquid nutritive medium. According to previously performed experiments it was proved that the selected isolate, originating from sunflower meal, had the ability to degrade these fusariotoxins when growing on the modified Vogel's agar supplemented with crude extracts of DAS and T-2 toxin.

In order to determine biodegradation of fusariotoxins, the liquid nutritive medium – SPY (5% sucrose + 0.1% peptone + 0.1% yeast extract, pH 6.2) was simultaneously inoculated with the isolate *M. racemosus* f. *racemosus* 1215/09 and: a) *Fusarium semitectum* SL-B (DAS producer) or b) *F. sporotrichioides* R-2301 (T-2 toxin producer). The SPY media, inoculated with single fungal isolates, were used as a control of toxin biosynthesis. The cultures were incubated at room temperature (21-26°C) on the rotary shaker (175 rpm). After the 3-5-day incubation, the filtration of liquid cultures and the extraction of fusariotoxins from filtrates with ethyl-acetate were performed. Determinations of DAS and T-2 toxin were done by thin layer chromatography using silica gel G.

Depending on the incubation duration, *M. racemosus* f. *racemosus* in the mixed culture with *F. semitectum* degraded from 90.0 to 99.97% of DAS present in the medium (40,000-120,000 µg l⁻¹), while in the mixed culture with *F. sporotrichioides* it degraded from 95.0 to 96.7% of T-2 toxin present in the medium (240,000 µg l⁻¹).

Sterile filtrates of mixed cultures and single culture of *M. racemosus* f. *racemosus*, obtained by passing liquid cultures through the 0.45-µm membrane filter and added to the SPY medium, did not affect degradation of type A trichothecenes that had been biosynthesised by isolates *F. semitectum* SL-B and *F. sporotrichioides* R-2301 in the liquid medium.

KEY WORDS: diacetoxyscirpenol (DAS), T-2 toxin, biodegradation, *M. racemosus* f. *racemosus*, liquid medium

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INTRODUCTION

Trichothecenes are a group of toxic secondary metabolites produced by several genera of *Fungi imperfecti* out of which the genus *Fusarium* is the most important. Some of trichothecenes are natural contaminants of cereal crops during their growth in fields or during grain storage under high environmental moisture. Since these compounds are toxic to both, humans and animals, it is of great interest for food and feed industry to prevent the introduction of fusariotoxins into the nutrition chain (Shi ma et al., 1997).

Despite of the application of different preventive measures that avoid the occurrence of *Fusarium* spp. and their toxins (crop rotation, ploughing, weed and insect control, appropriate sowing and harvest time, etc.), the obtained results are usually limited and unsatisfactory, hence it was necessary to develop procedures of detoxication. It is considered that biodegradation or biotransformation is the only efficient method of detoxication of mycotoxins that can only be poorly bound by various adsorbents (Rodrigues and Binder, 2008). This group of mycotoxins also includes type A trichothecenes (diacetoxyscirpenol – DAS and T-2 toxin) in which the 12,13-epoxy ring is responsible for their toxicity.

Studies carried out by Ueno et al. (1983) and Beeton and Bull (1989) point out to a possible role of soil bacteria in the transformation of T-2 toxins. Moreover, Beeton and Bull (1989) reported that certain natural bacterial communities or monocultures originating from soil and freshwater represented agents that could detoxify T-2 toxin and related trichothecenes.

Swanson et al. (1988) determined that intestinal bacterial biota originating from rats completely biotransformed T-2 toxin to its de-epoxy products (de-epoxy HT-2 and de-epoxy T-2 triol). The absence of complete de-epoxidation was recorded in experiments with chicken intestinal bacteria that degraded T-2 toxin and DAS exclusively by the process of deacetylation (Youn g et al., 2007). In contrast to this, intestinal and fecal microorganisms of rats, cattle and pigs completely biotransformed DAS to de-epoxy monoacetoxyscirpenol. Binder et al. (2000) isolated a new bacterial strain of the genus *Eubacterium* (strain BBSH 797) from a bovine rumen that had a potential to biodegrade epoxy group of trichothecenes to diene (Sca tzm a y r et al., 2006).

Beside bacteria there are other types of microorganisms that are capable of T-2 toxin biotransformation: protozoa (Kiesling et al., 1984), yeasts (Rodrigues and Binder, 2008), and mycobiota (Jesenska and Sajbidorova, 1991). Jesenska and Sajbidorova (1991) identified *Alternaria* sp., *Ulocladium* sp., *Aspergillus candidus* Link, *A. flavus* Link, *Cladosporium cladosporioide* (Fr.) G. A. de Vries and *C. macrocarpum* Preuss in the group of moulds that very rapidly degraded T-2 toxin (for 48 h).

Considering the natural presence of T-2 toxin worldwide in toxicologically relevant concentrations, as well as, its powerful toxicity, the aim of the present study was to investigate the capacity of biodegradation of T-2 toxin and related metabolite DAS under laboratory conditions in the liquid medium by use of the fungal isolate *Mucor racemosus* f. *racemosus* 1215/09.

MATERIAL AND METHODS

Microorganisms. The fungal isolate *Mucor racemosus* Fresen. f. *racemosus* 1215/09 obtained during the routine analysis of sunflower meal at the Department of Microbiology, Centre for Bio-Ecology, Zrenjanin, in 2009 was selected for tests of biodegradation of type A trichothecenes (DAS and T-2 toxin) having in mind our previous results (Bočarov - Stančić et al., 2010). The identification of fungal isolate was performed after Domsh et al. (1980).

The following two fungal isolates were selected for the production of type A trichothecenes in the experiments of biodegradation: a) *Fusarium semitectum* Berk. & Rev. SL-B, the Centre's isolate from the alfalfa hay, for previous studies proved that it had capability to biosynthesise DAS (Bočarov - Stančić et al., 2005) and b) *F. sporotrichioides* Sherb. R-2301, leg. Dr. D. Latus, Germany, the strain known as a good producer of T-2 toxin (Bočarov - Stančić et al., 2007). Fungal cultures were maintained on potato dextrose agar (PDA) at 4-6°C.

Inoculation of the liquid medium SPY (5% sucrose + 0.1% peptone + 0.1% yeast extract, pH 6.2) was done with five pieces (each 5 x 5 mm) of the fungal material taken from investigated isolates subcultured during seven days on PDA Petri plates at 27±1°C.

The first stage of the experiment encompassed studies on the ability of biotransformation of DAS and T-2 toxin by the fungal microorganism *M. racemosus* f. *racemosus* 1215/09. The variants were as follows: **I** – combination of *F. semitectum* SL-B and *M. racemosus* f. *racemosus* 1215/09, **II** – combination of *F. sporotrichioides* R-2301 and *M. racemosus* f. *racemosus* 1215/09. As a control of biosynthesis of fusariotoxins, the SPY medium was inoculated by single fungal cultures: **III** – *M. racemosus* f. *racemosus* 1215/09, **IV** – *F. semitectum* SL-B i **V** – *F. sporotrichioides* R-2301.

The second stage of the experiment included studies on capacities of extracellular enzymes of the monoculture *M. racemosus* f. *racemosus* 1215/09 (**III**), as well as mixed cultures of *F. semitectum* SL-B + *M. racemosus* f. *racemosus* 1215/09 (**I**) and *F. sporotrichioides* R-2301 + *M. racemosus* f. *racemosus* 1215/09 (**II**) to biodegrade type A trichothecenes. Sterile filtrates of cultures from the first stage of the experiment, obtained by passing through Minisart NML of 0.45 µm, in the amount of 6 ml, were added to the SPY medium immediately prior to inoculation with producers of DAS and T-2 toxin. The variants were as follows: *F. semitectum* SL-B + **I**, *F. semitectum* SL-B + **II**, *F. semitectum* SL-B + **III**, *F. sporotrichioides* R-2301 + **I**, *F. sporotrichioides* R-2301 + **II** and *F. sporotrichioides* R-2301 + **III**. As a control of biosynthesis of fusariotoxins, the SPY medium was inoculated by single fungal cultures: **IV** – *F. semitectum* SL-B and **V** – *F. sporotrichioides* R-2301.

Incubation conditions. A total of 100 ml of the semi-synthetic medium SPY placed in Erlenmeyer flasks (500 ml) were inoculated and incubated on a rotary shaker (175 rpm) at room temperature (21-26°C) during five and/or three days. All tests were performed in three replicates.

Sample preparation for the analysis of fusariotoxins. Liquid fungal mixed cultures, as well as monocultures, were filtered after incubation on the rotary shaker. Crude extracts of type A trichothecenes were obtained by use of ethyl-acetate. Further purification of DAS and T-2 toxin was carried out applying the procedure of Romer et al. (1978), while thin layer chromatography was performed after Pepljnjak and Babić (1991).

RESULTS AND DISCUSSION

The isolate *M. racemosus* f. *racemosus* 1215/09 was selected for the experiments of biodegradation of type A trichothecenes not only because of literature data showing that various species of the genus *Mucor* could biotransform these fusariotoxins (El-Sharkawy and Abbas, 1991) but primarily due to such capacity of the selected isolate was confirmed by our previous experiments (Bočarov-Stanić et al., 2010). During the growth of *M. racemosus* f. *racemosus* 1215/09 on the modified Vogel's agar (Vogel, 1956) to which crude extracts of DAS and T-2 toxins were added (300 µg/Petri dish) it completely biotransformed the stated fusariotoxins seven days after incubation at 27±1°C.

Tab. 1 – Biodegradation of DAS and T-2 toxin in the liquid medium by use of *Mucor racemosus* f. *racemosus* (SPY, 175 rpm, 25-26°C)

Fungus	Isolate design.	Days	Final pH	Toxin yield (µg l ⁻¹)		Yield reduction (%)
				DAS	T-2	
<i>Mucor racemosus</i> f. <i>racemosus</i>	1215/10	3	7.90	n.d. (<21)	n.d. (<21)	-
		5	8.20	n.d. (<21)	n.d. (<21)	-
<i>Fusarium semitectum</i>	SL-B	3	4.85	120,000	n.d. (<21)	-
		5	5.00	40,000	n.d. (<21)	-
<i>F. semitectum</i> + <i>M. racemosus</i> f. <i>racemosus</i>	SL-B + 1215/09	3	4.40	40	n.d. (<21)	99.97
		5	4.80	4,000	n.d. (<21)	90.0
<i>Fusarium sporotrichioides</i>	R2301	3	4.10	n.d. (<21)	240,000	-
		5	4.50	n.d. (<21)	240,000	-
<i>F. sporotrichioides</i> + <i>M. racemosus</i> f. <i>racemosus</i>	R2301 + 1215/09	3	3.90	n.d. (<21)	12,000	95.0
		5	4.45	n.d. (<21)	8,000	96.7

Significant differences in characteristics of the growth of fungal monocultures and mixed cultures were observed during their incubation in the liquid medium SPY (Table 1).

pH value. In case of single culture of *M. racemosus* f. *racemosus* 1215/09, the increase in pH in the SPY medium ranged from the initial value of 6.20 to the value of 7.90 and higher, while in case of single cultures of *F. semitectum* SL-B and *F. sporotrichioides* R-2301, as well as, mixed cultures of *M. race-*

mosus f. racemosus 1215/09 and these producers of fusariotoxins, pH values were reduced to 5.00 and below 5.00 during incubation in the liquid medium.

Somewhat higher pH values were observed in all combinations (mono- and mixed cultures) during prolonged incubation from 3 to 5 days.

Biosynthesis of type A trichothecenes. The isolate *M. racemosus f. racemosus* 1215/09 did not biosynthesised DAS and T-2 toxin under laboratory test conditions (Table 1).

The DAS yield in the *F. semitectum* SL-B monoculture was higher after 3-day ($120,000 \mu\text{g l}^{-1}$) than after 5-day incubation ($40,000 \mu\text{g l}^{-1}$) in the liquid semi-synthetic medium SPY on the rotary shaker.

On the other hand, the incubation duration under the same laboratory conditions (SPY, 175 rpm, 25-26°C), did not affect the amount of the produced T-2 toxin by use of single *F. sporotrichioides* R-2301 culture. The yield of the T-2 toxin was equal ($240,000 \mu\text{g l}^{-1}$) in both cases (after three and five days of incubation).

Biotransformation of type A trichothecenes. Biodegradation of DAS and T-2 toxin in the liquid medium by *M. racemosus f. racemosus* 1215/09 was tested by mixed cultures of this fungal isolate with *Fusarium* species that were producers of the same trichothecenes (Table 1).

In case of a mixed cultures of *F. semitectum* SL-B with *M. racemosus f. racemosus* 1215/09, the DAS yield was significantly lower after the 3-day inoculation ($40 \mu\text{g l}^{-1}$) than after 5-day incubation under the same *in vitro* conditions (Table 1). If these results are compared with the DAS yield in the single *F. semitectum* SL-B culture it is observable that *M. racemosus f. racemosus* 1215/09 biotransformed 90.0% of DAS present in the liquid medium after 5 days of incubation. The reduction of the yield of this type A trichothecenes was higher after 3-day incubation and amounted to 99.97%.

Similar results were also gained in case of mixed culture of *F. sporotrichioides* R-2301 with *M. racemosus f. racemosus* 1215/09 (Table 1). Although recorded differences in T-2 toxin yields after 3- ($12,000 \mu\text{g l}^{-1}$) and 5-day inoculation ($8,000 \mu\text{g l}^{-1}$) were not great, *M. racemosus f. racemosus* 1215/09 biodegraded over 90.0% of other type A trichothecene present in the liquid medium. The yield reduction of T-2 toxin in this mixed culture amounted to 95.0, i.e. 96.6% after 3-, i.e. 5-day-incubation, respectively.

According to El-Sarkawy and Abbas (1991) biotransformation of T-2 toxin by use of species *Aspergillus niger* van Tieg. and *Mucor mucedo* Fresen. was done by acetylation and deacetylation of this fusariotoxin, without degradation of the trichothecene skeleton, hence it is assumed that it also happened in experiments performed with our fungal isolate *M. racemosus f. racemosus* 1215/09.

A great capacity of the isolate *M. racemosus f. racemosus* 1215/09 to biodegrade DAS and T-2 toxin is the best observable if our results are compared with results gained by other authors. In the *in vitro* experiment of biotransformation of certain A- and B-trichothecenes with probiotic microorganisms (genera *Bacillus*, *Lactobacillus*, and *Saccharomyces*) Böhm et al. (2000) applied significantly lower concentrations of fusariotoxins. Those were con-

centrations that could be found under natural conditions and they amounted to 500 µg kg⁻¹ for both, DAS and T-2 toxin. However, the reduction in concentrations of tested trichothecenes was not recorded, and T-2 and DAS slowed down the growth of some strains of probiotic microorganisms.

Tab. 2 – Effects of filter sterilized liquid mono- and mixed culture *Mucor racemosus* f. *racemosus* on biosynthesis of DAS and T-2 toxin in the liquid medium (SPY, 3 days, 175 rpm, 21-23°C)

Fungus	Isolate design.	Added filtrate	Final pH	Toxin yield (µg l ⁻¹)		Yield reduction (%)
				DAS	T-2	
<i>Fusarium semitectum</i>	SL-B	-	4.76	34,000	n.d. (<21)	0
		I	4.55	34,000	n.d. (<21)	0
		II	4.75	34,000	n.d. (<21)	0
		III	4.70	34,000	n.d. (<21)	0
<i>Fusarium sporotrichioides</i>	R-2301	-	4.20	n.d. (<21)	184,000	0
		I	4.25	n.d. (<21)	184,000	0
		II	4.18	n.d. (<21)	184,000	0
		III	4.12	n.d. (<21)	184,000	0

Legend:

I - *F. semitectum* SL-B+*M. racemosus* f. *racemosus* 1215/09 (SPY 5 days, 175 rpm);

II - *F. sporotrichioides* R-2301+*M. racemosus* f. *racemosus* 1215/09 (SPY, 5 days, 175 rpm);

III - *M. racemosus* f. *racemosus* 1215/09 (SPY, 5 days, 175 rpm).

The decline of pH values, in the medium for DAS and T-2 toxin biosynthesis, from the initial 6.20 to the values ranging from 4.12 to 4.76 at the end of incubation (Table 2), was observed in liquid cultures of *F. semitectum* SL-B and *F. sporotrichioides* R-2301 with or without addition of sterile filtrates of monoculture of *M. racemosus* f. *racemosus* 1215/09 (**III**) or mixed cultures (**I** and **II**).

Extracellular enzymes of *M. racemosus* f. *racemosus* 1215/09 present in filtrates **I**, **II** and **III** did not essentially affect the changes in the pH value, nor the yield of the same fusariotoxins in tested producers of DAS and T-2 toxin after 3-day incubation in SPY. Similar results, i.e. absence of capacities of cell free supernatants of microbial cultures to transform one type B trichothecene (deoxynivalenol) were published by Völki et al. (2004).

CONCLUSION

The isolate *M. racemosus* f. *racemosus* 1215/09 biodegraded 99.97%, i.e. 90.0% of DAS present in the liquid medium SPY after 3-, i.e. 5-day incubation, respectively.

Under the same laboratory conditions the culture *M. racemosus* f. *racemosus* 1215/09 biotransformed 95.0%, i.e. 96.7% of T-2 toxin present in the medium after 3-, i.e. 5-day incubation, respectively.

Extracellular enzymes of the isolate *M. racemosus* f. *racemosus* 1215/09 were not capable to degrade tested type A trichothecens under laboratory conditions.

The gained results have to be considered just as a first step in the development of practically and commercially acceptable technology for decontamination of mycotoxin-containing food and feed .

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IN VITRO ДЕГРАДАЦИЈА ДИАЦЕТОКСИСЦИРПЕНОЛА И Т-2 ТОКСИНА ПОСРЕДСТВОМ ИЗОЛАТА *MUCOR RACEMOSUS* FRESEN. F. *RACEMOSUS*

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Резиме

У контролисаним *in vitro* условима проучавана је способност изолата *Mucor racemosus* f. *racemosus* 1215/09 да деградује трихотецене типа А (диацетоксисцирпенол - ДАС и Т-2 токсин) у течной хранљивој подлози. Претходним експериментима је доказано да одабрани изолат, пореклом са сунцокретевог сачме, поседује способност разградње наведених фузариотоксина, који су као сирови екстракти додати у модификовану Вогелову подлогу:

У циљу утврђивања биодеградације фузариотоксина течна хранљива подлога СПК (5% сахароза + 0,1% пептон + 0,1% екстракт квасца, рН 6,2) је засејана

у исто време изолатом *M. racemosus* f. *racemosus* 1215/09 и: а) *Fusarium semitectum* SL-B (произвођач ДАС-а) или б) *F. sporotrichioides* R-2301 (произвођач Т-2 токсина). Као контрола биосинтезе токсина коришћена је СПК подлога инокулисана појединачним изолатима гљива. Културе су инкубиране на ротационој тресилици (175 о/мин) током 3-5 дана на собној температури (21-26°C). Након 3 до 5 дана инкубације вршено је филтрирање течних култура и екстракција фузариотоксина из филтрата етил-ацетатом. Детерминација ДАС-а и Т-2 токсина је рађена танкослојном хроматографијом на силика гелу Г.

Зависно од дужине инкубације, *M. racemosus* f. *racemosus* је у здруженој култури са *F. semitectum* деградовала 90,0-99,97% ДАС-а присутног у подлози (40.000-120.000 µg l⁻¹), док је у здруженој култури са *F. sporotrichioides* разградила 95,0-96,7% Т-2 токсина присутног у подлози (240.000 µg l⁻¹).

Стерилни филтрати мешаних култура и појединачне културе *M. racemosus* f. *racemosus*, добијени пропуштањем течних култура кроз 0,45 µm мембрански филтер и додати СПК подлози, нису утицали на разградњу трихотецена типа А које су биосинтетисали изолати *F. semitectum* SL-B и *F. sporotrichioides* R-2301 у течной подлози.