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# THE ROLE OF *PAECILOMYCES LILACINUS* (THOM) SAMSON AND OTHER FUNGAL SPECIES IN BIODEGRADATION OF OCHRATOXIN A

ABSTRACT: Nine isolates of fungi of genera *Aspergillus, Fusarium*, *Paecilomyces* and *Penicillium* were cultured on the modified Vogel's medium with the addition of crude ochratoxin A (OTA) extract. This crude OTA extract was derived from a natural solid substrate on which *Aspergillus ochraceus* strain CBS 108.08 was cultivated. OTA was isolated, partially purified, dried by evaporating and dissolved in ethanol (1 mg $ml^{-1}$), and added to the test medium up to the final concentration of 10 µg $ml^{-1}$. The presence of OTA residues was determined after 7 and 14 day cultivation of fungi in the test medium at 27±1°C. The *Paecilomyces lilacinus* isolate (Inf. 2/A), which completely degraded OTA (150 µg) after only seven days, was selected for further studies. Wet sterile rice grains (50 g + 25 ml distilled water) were inoculated with individual isolates of fungi *A. ochraceus* (strain CBS 108.08) and *P. lilacinus* (isolate Inf. 2/A), and with their combination. In the case of *P. lilacinus* monoculture, 0.9 mg of crude OTA was also added into cultivation substrate. Each test was done in three replications. After the four week cultivation of individual and combined fungi at 27±1°C, inoculated rice grains were dried to the constant weight and pulverized. OTA was determined in these samples by the application of standard TLC method for fodder analysis.

OTA in the amount of 61.310 µg $kg^{-1}$ dry matter (DM) was determined only in the samples inoculated with a producer of ochratoxin A (*A. ochraceus*, strain CBS 108.08). On the other hand, a much smaller amount of OTA (80 µg $kg^{-1}$ DM) was detected in samples inoculated with combined cultures of *A. ochraceus* and *P. lilacinus* isolates. Gained results indicate that *P. lilacinus* degraded, on average, 99.8% of OTA. After four week cultivation, the same fungal isolate in the samples of wet sterile rice kernels with the addition of 0.9 mg of crude OTA, completely degraded added crude OTA (<8 µg $kg^{-1}$).

KEY WORDS: biodegradation, ochratoxin A, *Paecilomyces lilacinus*

## INTRODUCTION

Ochratoxin A (OTA) is considered to be one of the most toxic mycotoxins, whose presence has been established in food, feed and commercial feed

mixtures. In order to reduce the presence of this mycotoxin in food, different methods for the inhibition of the growth of OTA producing fungi (*Aspergillus alliaceus,* T h o m and C h u r c h*, A. Carbonarius,* B a i n, *A. Ochraceus,* W i l h e l m*, A. steynii,* F r i s and S a m s o n*, A. westerdijkiae,* F r i s and S a m s o n*, Penicillium nordicum,* D r a g o n i and C a n t o n i, and *P. Verrucosum,* E i e r c h x) and for the prevention of this mycotoxin production are applied (A b r a n h o s a et al., 2010). Biological methods have been considered as an alternative to physical and chemical treatments.

Numerous microorganisms capable of degrading, absorbing and detoxifying OTA and ochratoxin B (OTB) have been reported in the literature. Besides several protozoan species, yeasts and bacteria, filamentous fungi are also believed to have these abilities. It is presumed that two biochemical pathways may be involved in this process (K a r l o v s k y, 1999). First, OTA can be biodegraded through the hydrolysis of amide bond to the non-toxic compounds of L-*β*-phenylalanine and OTα. Secondly, a more hypothetical process, involves OTA being degraded via the hydrolysis of lactone ring, although in this case, the final degradation product is an opened lactone form of OTA, which is of similar toxicity to OTA when administered to some laboratory animals (A b r a n h o s a et al., 2010).

By the application of ochratoxin biodegradation it is possible to avoid toxic effects, primarily nephrotoxicity and carcinogenicity of ochratoxin, when found in food and feed in the amounts that can be toxic to both humans and animals. Therefore, finding new candidates of microorganisms, especially fungi of various geographical and agroecological origins, which will be more efficient in biodegradation of ochratoxins and bioremediation of food, is of a great interest. Accordingly, the aim of this study was to determine the role of some fungi, originating from Serbian region and not expressing toxigenic properties, in the biodegradation of OTA.

## MATERIALS AND METHODS

*Microorganisms*. Nine isolates of fungi belonging to species *Aspergillus flavus* (Bain. and Sart.) Thom and Church (2), *A. fumigatus* Fres. (1), *A. ochraceus* Wilhelm(1), *Fusarium poae* (Peck) Wollenw. (1), *Fusarium* sp. (1), *Paecilomyces lilacinus* (Thom) Samson (1) and *Penicillium* spp. (2) were selected as test organisms. With an exception of the culture of *A. ochraceus* strain CBS 108.08, which is a known ochratoxin A producer (B o č a r o v-S t a n č i ć et al., 2009b), tested fungi originated from Serbian samples of livestock feed and their components not contaminated by mycotoxins, or were isolated from air in the course of a regular sterility control of premises in which microbiological analyses were carried out. The fungal identification was performed after D o m s h et al. (1980) and S a m s o n and v a n R e e n e n - H o e k s t r a (1988). The fungal cultures were kept on potato dextrose agar (PDA) at 4-6ºC.

*Crude toxin production*. Crude ochratoxin A (OTA) was produced by the isolate of *A. ochraceus* strain CBS 108.08 using a procedure described in detail

in the previous manuscript written by B o č a r o v-S t a n č i ć et al. (2009b). Inoculated Roux bottles containing 50 g of sterilized wheat kernels, wetted with 50 ml of sterile water, were cultivated at 30±1ºC for four weeks. Samples obtained after the cultivation were dried for 24 h or more at 60ºC until constant weight. After the pulverization of dried samples, crude OTA was obtained by the use of Serbian official methods for sampling and fodder analyzing (The O f f i c i a l G a z e t t e of S F R Y, issue 15/87). When the chloroform extract of OTA was evaporated, dry residue of this mycotoxin was dissolved in 96% ethanol (1 mg $ml^{-1}$) and stored until used at 4-6ºC.

*Cultivation conditions*. Test fungi were cultured on the modified Vogel's medium N (pH 6.3) with the addition of crude OTA extract for 14 days at 27±1ºC. OTA was added to the test medium immediately before its pouring into Petri dishes (15 ml per dish), and its final concentration in the medium amounted to 10 μg $ml^{-1}$. A test microorganism was applied with an inoculating loop to the central part of the solidified medium. The modification of Vogel's minimal medium (V o g e l, 1956) consisted of excluding the solution of biotin and sucrose, and addition of peptone (1 g $l^{-1}$) and yeast extract (2 g $l^{-1}$).

*Mycotoxicological studies*. Fungal capacity to produce and degrade OTA was preliminary studied by a rapid screening method described by F i l t e n b o r g et al. (1983), and modified by B o č a r o v-S t a n č i ć et al. (2009a, 2010). Vogel's minimal medium without addition of mycotoxins was used only in case of studies on fungal ability to biosynthesize OTA.

The second part of the experiment encompassed the study on the capability of the selected *Paecilomyces lilacinus* isolate (Inf. 2/A) to biodegrade OTA. Wet sterile rice grains (50 g + 25 ml distilled water) were inoculated with individual isolates of fungi *A. ochraceus* (strain CBS 108.08) and *P. lilacinus* (isolate Inf. 2-A) and with their combination. In the case of *P. lilacinus*, crude ochratoxin A (0.9 mg) was added to the cultivation substrate. After the four week cultivation of individual and combined fungal cultures at 27±1°C, inoculated grains were dried to the constant weight (dry residue) and pulverized. OTA in these samples was determined by the application of standard thin layer chromatography (TLC) method for the fodder analysis (T h e O f f i c i a l G a z e t t e o f S F R Y, issue 15/87). All tests were performed in three replications.

Thin layer chromatography was done in saturated system of the benzene-acetic acid mixture (9:1, v/v). OTA was visually detected under long wave UV rays (366 nm) after TCL plates were sprayed with $NaHCO_3$ solution in ethanol and heated for 10 min at 130°C. The limit of detection (LOD) of the applied TLC method amounted to 8 μg $kg^{-1}$.

## RESULTS AND DISCUSSION

Out of nine fungal isolates tested by a rapid screening method (F i l t e n b o r g - a et al., 1983; B o č a r o v-S t a n č i ć et al., 2009a, 2010), only the isolate of *A. ochraceus* (CBS 108.08) produced OTA in Vogel's minimal me-

dium without the addition of this mycotoxin (Table1). In previous studies, this isolate proved to be a good producer of OTA (B o č a r o v-S t a n č i ć et al., 2009b).

Tab. 1 – Capability of biosynthesis (control test for toxigenicity) and microbiological degradation of OTA by means of fungi

| Ord. no. | Species | Isolate origin | Isolate design. | Biosynthesis[a] Degradacija[a] | | Degradation[b] | |
|---|---|---|---|---|---|---|---|
| | | | | 7 d. | 14 d. | 7 d. | 14 d. |
| 1. | *Aspergillus flavus* | Soya bean grits | 675/09 | no | no | no | no |
| 2. | *A. flavus* | Air | D-2 | no | no | no | no |
| 3. | *A. fumigatus* | Air | D-3 | no | no | no | no |
| 4. | *A. ochraceus* | CBS | 108.08 | no | yes | no | no |
| 5. | *Fusarium poae* | Wheat kernel | 598/09-8 | no | no | no | no |
| 6. | *Fusarium* sp. | Air | Inf. 3 | no | no | no | no |
| 7. | *Paecilomyces lilacinus* | Air | Inf. 2/A | no | no | yes | yes |
| 8. | *Penicillium* sp. | Air | Inf. 2/B | no | no | no | no |
| 9. | *Penicillium* sp. | Wheat kernel | 598/09-7 | no | no | no | no |

[a] Vogel's minimal medium without addition of OTA,
[b] Vogel's minimal medium with addition of OTA.

*P. lilacinus* isolate (Inf. 2/A) was a single isolate that biotransformed the total amount of crude OTA (150 μg per Petri dish) after only seven days under given laboratory conditions (Table 1). Although numerous available literature data show that fungi, such as *Aspergillus clavatus* Desm., *A. ochraceus, A. versicolor* (Vuill.) Tirab., *A. wenti* Wehmer (A b r a n h o s a et al., 2002), *A. niger* Tiegh., *A. japonicus* Saito (B e j a o u i et al., 2006), *Rhizopus microsporus* Teigh., *R. homothallicus* Hesseltine & Ellis, *R. oryzae* Went and Prinsen-Geerligs (V a r g a et al., 2005) and others, can biotransform up to 95% of the initial OTA amounts, in this study, our own isolates of the genus *Aspergillus* were not capable of degrading this mycotoxin. On the other hand, we could not find in literature at our disposal that *Paecilomyces* spp. was capable of OTA detoxification.

It is interesting to point out that the fungus *P. lilacinus* (Inf. 2/A), as well as *A. flavus* (D-2), *A. fumigatus* (D-3), *Fusarium* sp. (Inf. 3) and *Penicillium* sp. (Inf. 2/B), was isolated after screening ambient air above working areas in the Department of Microbiology of the Bio-Ecological Center in Zrenjanin. Although all these isolates grew well on Vogel's minimal medium, to which crude OTA had been added, only *P. lilacinus* isolate (Inf. 2/A) had capability to biodegrade this mycotoxin.

The amount of OTA (150 μg per Petri dish), used in this experiment for the growth of the test organisms, was significantly higher (Table 2) than the common natural contamination of different substrates with this mycotoxin. In similar experiments of OTA biodegradation, other authors used 40 μg (H w a n g

and D r a u g h o n, 1994) or 50 μg of this mycotoxin (B ö h m et al., 2000), which is three-fold lower amount than that used in our experiment. The obtained preliminary results point out that our *P. lilacinus* isolate (Inf. 2/A) has an excellent potential for OTA biotransformation.

After the completed cultivation of *P. lilacinus* (Inf. 2/A) on wet sterile rice grains, the result obtained on Vogel's minimal medium was confirmed – this fungi was not toxigenic because it had no ability to biosynthesize OTA (Table 2).

Tab. 2 – Amount and percentage of OTA biodegradation (average values) after the four week cultivation on wet sterile rice grain

| No. | Species | Isolate/isolates | Dry residue (g) | Amount of OTA (μg $kg^{-1}$) | Degraded OTA (%) |
|---|---|---|---|---|---|
| | *P. lilacinus* | Inf. 2/A | 33.13 | n.d. (<8) | 0 |
| | *P. lilacinus*[a] | Inf. 2/A | 32.95 | n.d. (<8) | 100.0 |
| | *P. lilacinus* + *A. ochraceus* | Inf. 2/A+ CBS 108.08 | 27.00 | 80 | 99.8 |
| | *A. ochraceus* | CBS 108.08 | 16.13 | 61,310 | – |

[a] wet sterile rice grain with the addition of 0.

It was not possible to detect OTA residues (<8 μg $kg^{-1}$) in the samples of wet sterile rice grains to which 0.9 mg of crude OTA was added, and which were inoculated only with *P. lilacinus* isolate (Inf. 2/A), because this isolate completely biotransformed the added amount of crude OTA. If this mycotoxin was not degraded, the expected amount of it would be 27.160 μg $kg^{-1}$ dry matter (DM).

V a r g a et al. (2005) observed effective degradation of OTA in wheat kernels that were inoculated with *Rhizopus stolonifer* (Ehrenb.) Lind. The same authors also observed that this and other species of the same fungal genus (*R. microsporus, R. homotallicus* and *R. oryzae*) were capable to biotransform OTA in the liquid medium (up to 95% of initial quantity). However, the biodegradation process in the liquid medium was much faster than in wheat grain (max. 16 days in comparison to four weeks).

In the samples of wet sterile rice kernels inoculated only with *A. ochraceus* (CBS 108.08), detected amount of dry residue (16.13 g) was lower than in the medium inoculated with *P. lilacinus* (33.13 g) (Table 2). The addition of 0.9 mg of crude OTA into the rice grain used for *P. lilacinus* monoculture did not significantly affect the amount of dry residue in *P. lilacinus* (32.95 g). After the four week cultivation of both fungi combined on the given substrate, the amount of dry residue was 27.0 g. The reduction of the dry residue was not surprising considering the competition for the same substrate between *A. ochraceus* and *P. Lilacinus* (Table 2).

Significantly lower average amount of OTA (80 μg $kg^{-1}$DM) was established in the samples of wet sterile rice kernels inoculated with combined cultures of *A. ochraceus* and *P. lilacinus*, than that detected in *A. ochraceus*

monoculture (61.310 μg $kg^{-1}$ DM). These results indicate that *P. lilacinus* isolate degraded, on average, 99.8% OTA during the four week cultivation on the solid natural substrate.

The determined percentage of biotransformation of OTA in the present study was significantly higher than that established by E n g e l h a r d t (2002), who observed capability of degradation of OTA and OTB in three fungal species cultured on barely kernels. According to this author, the white rot fungus *Pleurotus ostreatus* (Jacq. ex Fr.) Kumm. was ranked first as it degraded 77 and 97% of the initial amount of OTA and OTB, respectively, after the four week cultivation. Achieved results point out that our *P. lilacinus* isolate has an excellent potential for biotransformation of OTA when grown not only in minimal Vogel's medium but also in rice grain substrate.

## CONCLUSION

Only *P. lilacinus* isolate (Inf. 2/A), out of nine fungal isolates tested by the rapid screening method, showed capability to biotransform OTA when grown during seven days at 27±1°C on Vogel's minimal medium with the addition of crude OTA (150 μg per Petri dish).

After four week cultivation at 27±1°C, the same isolate in samples of wet sterile rice kernels with the addition of 0.9 mg of crude OTA, completely degraded initially added ochratoxin (<8 μg $kg^{-1}$).

*P. lilacinus* (Inf. 2/A) grown together with a good OTA producer, *A. ochraceus* CBS 108.08, biodegraded 99.8% of OTA after the four week cultivation on wet sterile rice kernels at 27±1°C.

The isolate of fungus *P. lilacinus* (Inf. 2/A) has a significant potential for biotransformation of OTA, hence further studies will be aimed at finding the mode to use this isolate in safe decontamination of cereals and their products intended to be used as food and feed.

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# УЛОГА *PAECILOMYCES LILACINUS* (THOM) SAMSON И ДРУГИХ ВРСТА ГЉИВА У БИОДЕГРАДАЦИЈИ ОХРАТОКСИНА А

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Резиме

Девет изолата гљива из родова *Aspergillus, Fusarium*, *Paecilomyces* и *Penicillium* гајено је на модификованој Вогеловој подлози са додатком сировог екстракта охратоксина А (ОТА). Сирови екстракт ОТА је добијен из чврстог природног супстрата на којем је гајен сој *Aspergillus ochraceus* CBS 108.08. Изолован и делимично пречишћен ОТА, упарен до сувог остатка и растворен у етанолу (1 mg $ml^{-1}$), додат је у тест подлогу до финалне концентрације 10 μg $ml^{-1}$. Након седам и 14 дана гајења култура гљива у тест подлози на 27 ± 1°C детерминисано је присуство резидуа ОТА применом модификоване методе F i l t e n b o r g -а и сар. (1983).

Од девет тестираних изолата за даља испитивања је одабран изолат *Paecilomyces lilacinus* (Inf. 2/A), који је већ после седам дана у потпуности разградио иницијалну количину ОТА (150 μg).

У другом делу експеримента влажно стерилно зрно пиринча (50 g + 25 ml дестиловане воде) засејано је са појединачним изолатима *A. ochraceus* (CBS 108.08) и *P. lilacinus* (Inf. 2-A), као и комбинацијом оба изолата. У случају монокултуре *P. lilacinu*s у подлогу је додат и сирови ОТА (0,9 mg). Сваки од тестова је урађен у 3 понављања. Након четири недеље гајења монокултура и мешаних култура гљива на 27±1°C, инокулисана зрна су осушена до константне тежине и самлевена до финог праха. У овим узорцима извршена је детерминација ОТА применом стандардне методе танкослојне хроматографије за анализу сточне хране.

У узорцима који су били засејани само са продуцентом ОТА (*A. ochraceus*, сој CBS 108.08) детектован је ОТА у просечној количини од 61.310 μg $kg^{-1}$ сувог остатка. У узорцима који су били засејани комбинованим културама изолата *A. ochraceus* и *P. lilacinus* утврђена је знатно мања просечна количина ОТА (80 μg $kg^{-1}$). Ови резултати указују да је изолат *P. lilacinus* разградио просечно 99,8% ОТА присутног у подлози за култивацију. У узорцима влажног стерилног зрна пиринча са додатком 0,9 mg сировог ОТА исти гљивични изолат је после четири недеље култивације комплетно биоразградио додат сирови ОТА (<8 μg $kg^{-1}$).