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Characterizing Ochratoxin A Production in *Aspergillus* Section Nigri: *A. foetidus var. pallidus* as an Emerging Contributor

Nermin Sarıgül *

Burdur Mehmet Akif Ersoy University, Science and Literature Faculty, Molecular Biology and Genetics Department, Burdur 15010, Turkey

Email: sarigul@mehmetakif.edu.tr

Abstract

Mycotoxins are toxic secondary metabolites produced by fungi, posing significant health risks to humans and animals. Among these, ochratoxins, primarily produced by *Aspergillus* and *Penicillium* species, are recognized for their nephrotoxic and carcinogenic properties. This study investigates the growth and ochratoxin A (OTA) production of *Aspergillus* Section Nigri species isolated from grapes in the Aegean region of Turkey. The Agar Plug and Rice Culture methods were employed to assess OTA production. While *Aspergillus carbonarius* is widely known as the primary producer of OTA, the study also identified that 27% of *Aspergillus foetidus var. pallidus* strains produced OTA, with two strains exceeding 750 ppb in production. The Agar Plug method was found to be faster and more efficient compared to the Rice Culture method, with both methods yielding satisfactory recovery rates. These findings highlight the role of environmental factors in OTA production and suggest that *A. foetidus var. pallidus* could also significantly contribute to OTA contamination, emphasizing the need for effective management strategies in agriculture and food safety.

Keywords: Ochratoxin A; *Aspergillus*; agar plug method; rice culture; HPLC.

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* Corresponding author.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi that cause toxic responses, leading to acute and chronic illnesses in humans and animals when consumed. The contamination of grains, fruits, and vegetables with mycotoxins poses global economic and health risks. Over 400 mycotoxins have been identified, but only 30 of them are considered harmful [1, 2]. Aflatoxins, ochratoxins, fumonisins, trichothecenes, patulin, citrinin, and zearalenone are among the commercially significant mycotoxins [3].

Ochratoxins are a group of mycotoxins produced primarily by species of *Aspergillus* and *Penicillium* fungi. Ochratoxin A (OTA) is the most studied and toxic member of this group. These mycotoxins commonly contaminate various food products, including cereals, coffee, dried fruits, wine, and spices, particularly in warm and humid conditions. OTA is known for its nephrotoxic effects, which can cause kidney damage in both humans and animals. It is also considered a potential carcinogen by the International Agency for Research on Cancer (IARC), classified as Group 2B, indicating it is possibly carcinogenic to humans. Additionally, OTA exhibits immunosuppressive, teratogenic, and hepatotoxic properties, posing significant health risks [4, 5, 6].

Ochratoxin contamination in food and feed depends on factors such as improper storage conditions, incorrect agricultural practices, and climate. Setting limit values for food and feed encourages producers to adopt better and more careful production methods, while also raising consumer awareness to avoid such products. At this point, the method chosen to detect the presence of ochratoxin in products becomes crucial. Rapid, simple, and cost-effective methods are advantageous [5, 7].

This study that is carried out under in vitro conditions aims to investigate the growing rate and ochratoxin production capacity of potentially ochratoxigenic species of *Aspergillus* Section Nigri which dominantly exist on grapes. Two different methods “Agar Plug Metod” and “Rice Culture Method” are used to determine the ochratoxin production capacity of these fungi.

2. Materials and Method

Potential ochratoxigenic 110 black *Aspergillus* strains, isolated from vineyards in Aegean Region Turkey, were obtained from previous studies.

2.1. Growth rate

Malt extract broth medium was used for growing mycelia. 100 ml of the medium was inoculated with approximately 10^6 spores and incubated at 120 rpm, 25 ^oC for 10 days. Mycelia were taken out daily and harvested by filtration. The amount of dry biomass was determined gravimetrically after the washed mycelia were dried at 105 ^oC to a constant weight.

2.2. Ochratoxin A production

OTA production were performed by cultivating the fungal strains in YES agar medium 25 ^oC for 3 days.

Agar plug method: For each strain three agar plugs were picked up from the central area of the colony. Each plug was weighed, mixed with 1 ml methanol and incubated for 1 h at room temperature before centrifuging at 8000 rpm for 10 min. The supernatant was filtered through 0,45 µm filters for HPLC analysis.

Rice culture (FDA) method: The FDA method is the official method to study the capacity to produce OTA by moulds []. Rice culture was placed into warring blender. 200 ml of 60% acetonitrile/water were added and stirred for 2 min. at high speed. The extract was filtered through a Whatman No 4 filter paper. 4 ml of filtrate were diluted with 44 ml of phosphate buffered saline and passed through immunoaffinity columns (Ochraprep, R-Biopharm Rhone). The column was washed with 20 ml PBS. The elution was done with 1,5 ml methanol for HPLC analysis.

2.3. HPLC

A high-performance liquid chromatographic method (HPLC) with fluorescent detector is used for the determination of OTA. A mobile phase consisting of acetonitrile:water:acetic acid (99:99:2, v/v/v) was used for the resolution of the compound on a C18 Hychrom IN ODS column (4,6x100mm; 5 µm). The excitation and emission wavelengths were λ_{ex}=333 nm and λ_{em}= 477 nm and the flow rate were 1ml/min. The injection volume was 20 µl and the retention time was around 6 min. The OTA levels were obtained based on the HPLC flourimetric response compared to standart (OTA, R-Biopharm Rhone).

3. Results

Growth rates of some ochratoxigenic fungi are shown in Figure 1. All the examined black *Aspergilli* strains completed their logarithmic phase within 3-4 days, indicating that secondary metabolite production begins at the end of this period. To determine the recovery of ochratoxin from the production medium, ochratoxin was added to randomly selected samples, and the recovery rate was calculated. Table 1 provides the recovery rates.

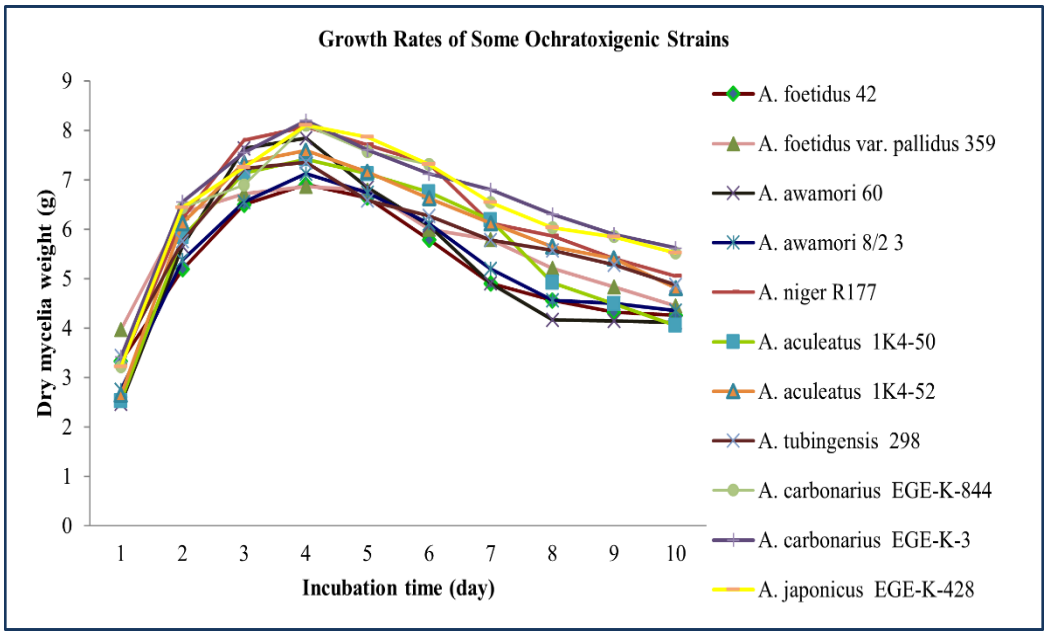

Figure 1: Growth curve of ochratoxigenic fungi

In this study, ochratoxin production was investigated using both standard rice culture and agar plug methods. Growth curves in liquid medium with malt extract broth showed that the logarithmic phase was completed on the 3rd or 4th day. Since secondary metabolite production occurs at the end of the logarithmic phase, toxin detection in the agar plug method was performed on the 3rd day. Furthermore, extending the incubation period in this method significantly increased sporulation, making detection via HPLC more challenging. It was observed that ochratoxin production varies depending on temperature, with strains naturally found in warm climates producing more OTA under such conditions [8]. However, in this study, the temperature was kept constant at 25 ^{o}C.

Standard OTA production and extraction using rice culture is more time-consuming and labor-intensive. With the agar plug method, extraction was performed directly from samples taken from the medium. This method is faster and easier compared to rice culture. When the two methods are compared, the recovery rate is satisfactory, making the agar plug method particularly preferable in cases with a high number of samples.

Table 1: Spikes recovery rates

Spiked OTA amount (ppb)	%R Agar Plug Method	%R Rice Culture
100	77.27±0.18	89.4±0.07
125	94.96±0.12	98.2±0.06
150	98.59±0.13	99.1±0.08

As a result of the study, ochratoxin A was detected in 36 out of 110 strains (Table 2). Of the 36 strains identified as producers, only 3 produced high levels of OTA. Considering that this study was conducted under confirmed optimal production conditions, it can be inferred that the number of producer strains in food and feed is low. For these few producers to pose significant risks, high levels of production would need to occur. Various studies have indicated that *Aspergillus carbonarius* is the primary producer of OTA [8-9-10-11]. All *Aspergillus carbonarius* strains tested produced OTA.

In our study, very low levels of OTA production were detected in *Aspergillus niger,* which is consistent with previous findings [12]. Although *Aspergillus niger* did not initially draw attention as an OTA producer, its role in producing another mycotoxin, fumonisin, is intriguing [13, 14, 15, 16]. This is particularly significant because *Aspergillus n*iger is classified as a GRAS organism and holds industrial importance. Studies have highlighted the OTA and especially fumonisin production by industrial strains of this species.

Aspergillus foetidus var. pallidus strains, which have black spores and belong to the nigri aggregate, exhibited approximately 27% OTA production. Additionally, two *Aspergillus foetidus var. pallidus* strains were the highest OTA producers among those tested.

Table 2: Number of ochratoxin producing strains

OTA amount (ppb)	***A. niger***	***A. carbonarius***	***A. japonicus***	***A. foetidus var. pallidus***
Not Detected	6	0	0	68
0-0.5	5	0	0	17
0.5-1.9	0	1	3	1
2.0-4.9	0	1	0	0
5.0-9.9	0	0	0	1
10.0-24.9	0	1	0	1
25.0-49.9	0	0	0	2
50.0-124.9	0	0	0	0
125.0-249.9	0	0	0	0
250.0-499.9	0	1	0	0
500.0-749.9	0	0	0	0
750.0-999.9	0	0	0	1
1000.0-1250.0	0	0	0	1
Total	11	4	3	92

4. Conclusions

Results showed that, *A. niger* aggregate and *A. carbonarius* strains can grow and produce OTA in 3-4 days and can be defined as fast growing fungi. Fast growth and OTA production properties should be considered for risk management: Region: The climate of the Aegean Region has a Mediterranean climate with hot, dry summers and mild to cool, wet winters. Dry summers may increase the spread of fungal spores and may increase OTA risk. Previous Crop and Inoculum: Crop residue on the soil surface is the major source of inoculum. Complete burial of debris by ploughing reduces the risk. Rainfall: Wet weather promotes fungal development and mycotoxin production. *Aspergillus niger* is an industrially important producer microorganism. Genes related to OTA production were determined in the genome analysis of the industrial strains used [17]. This situation creates the risk of toxicity in the products produced. Methods recommended to prevent toxin production include controlling the carbon sources used and environmental conditions such as pH and temperature [18, 19, 20, 21, 22]. The approach other than blocking production is detoxification. For this purpose, it is aimed to degrade OTA by using lactic acid bacteria, actinomycetes and other microorganisms [23, 24, 25, 26].

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