The Open Biotechnology Journal

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RESEARCH ARTICLE

The Production of Aflatoxins and Ochratoxin-A by *Aspergillus* Strains Isolated from Rice: Under *In vitro* Conditions

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Abstract:

Background:

Rice supplies a significant proportion of nutritional needs around the world. The fungal species that cause severe contamination of rice grains have created a major challenge to ensuring food safety.

Methods:

Thus, This study adopts an appropriate local method using potato dextrose agar (PDA) and thin-layer chromatography (TLC) for the production of Aflatoxins and Ochratoxin-A in Aspergillus flavus (NRRL strain 2999) and A. acrasus (NRRL strain 7431), receptively.

Results:

Promising early results suggest that an optimum protocol for the production of mycotoxin includes a temperature of 28°C for 21 d incubation. The average levels of A. flavus and A. acrasus were 625 and 482.67 µg/g, respectively, by comparing the fluorescence with the standard. As a result, a new and rapid method using PDA as a culture medium and TCL was developed to produce mycotoxins in rice from the Persian market.

Conclusion:

This study provides a novel (optimum) mechanistic approach concerning mycotoxins production from fungal species that could improve quality and ascertain its safety either in the field or in storage.

Keywords: Mycotoxin, Production, Culture media, Thin-layer chromatography, Persian market, Aflatoxin, Ochratoxin-A.

Article History	Received: January 29, 2023	Revised: July 10, 2023	Accepted: July 25, 2023

1. INTRODUCTION

Rice (*Oryza sativa*), being the second most consumed cereal worldwide, constitutes an important source of potential contamination by fungal pathogens [1, 2], and mycotoxin pollution is wide spread in this strategic food.

Mycotoxins are secondary fungus metabolites that induce chronic and acute toxic effects in animals and humans [3], with physiological, biochemical, and pathological changes by disrupting nutrient digestion, absorption, and metabolism [4]. Meanwhile, Aflatoxins (AFs) and Ochratoxin A (OTA) produced by Aspergillus strains are extremely carcinogenic mycotoxins [5], which are capable of causing mycotoxicosis (disease and death) [6]. During the last decades, the AFs and OTA are mycotoxins of major significance and hence there has been important research on a broad range of analytical and detection approaches that could be practically and useful [7]. These toxins have attracted great concern due to their adverse effects and agroeconomic significance [8]. Thus, the reduction of mycotoxin contamination in rice as well as estimating average dietary exposure seems to be necessary.

Various reputable Persian articles from different publishers as reference sources reported that some of the rice spices in the local market are imported from abroad and thus could be contaminated with fungal species [9 - 11]. On the other hand, a variety of analytical ways have been used to determine harmful fungi and the production of mycotoxins in rice. However, AFs and OTA have been detected in rice, but there is no valid published data to achieve an appropriate method for the production of these toxins under *in vitro* conditions. This study provides information about mycotoxin production in rice, thus could lead to improved quality and ascertaining its safety.

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2. MATERIALS AND METHODS

Aspergillus strains used in this study were acquired from the India Chandigarh Gene Bank for the production of AFs and OTA in *A. flavus* (NRRL strain 2999) and *A. acrasus* (NRRL strain 7431) in rice, receptively.

2.1. Preparation of Culture Media

To culture *Aspergillus* strains, a new (optimum) method using potato dextrose agar (PDA) as a culture medium was developed. First, we poured 9.75 g amount of the PDA culture medium (Potato Dextrose Agar) inside an Arlan, and mixed it with a certain amount of distilled water on the flame until the solution color became clear and reached the boiling temperature. Then, its cap is sealed completely with non-absorbent cotton and it is covered with aluminum foil and placed in an autoclave for 20 minutes at 121°C and thepsi 15 pressure. Later, we transferred the PDA culture medium in a sterile laminar hood along the flame to glass plates, after cooling the medium, the plates containing the culture medium were placed in incubator at 28°C for 24 hours to detect if the culture medium is contaminated.

2.2. Mycotoxins Production

After 24 hours, the culture mediums which were located inside the incubator and had no contamination were used for cultivating fungus. Then, the laminar hood was disinfected with alcohol cotton next and UV light was used for about 30 minutes, until the laminar hood medium became completely sterile. After preparing the laminar hood medium along with the flame, we cultivated the main culture of Aflatoxin and Acrotoxinproductive fungus by Anceas a spotty or grassy medium that we had prepared.Then, we tightly closed the plates with the paraffin strip and placed it in the incubator at a temperature of 28°C for 14 days to grow Aflatoxin and Acrotoxin fungus and produce spores .

First, 25 gram of half-grain rice (it should be half-grain rice, so that the fungus solution cultivated on rice is completely spread throughout the rice and the fungus can use all parts of the rice) were poured inside the 250ml Arlene and 24ml of distilled water was added to it, then we held the Arlene door with a cotton bung, next, we started shaking Arlene so that all rice inside the Arlene is completely soaked, then we placed Arlene containing soaked rice at room temperature for 2 hour, and after that left it for 20 minutes at 121°C and 15 psi pressure in the autoclave. In the end, we removed the Arlene containing rice and left it at room temperature for 24 hours.

2.2.1. Preparation of Essential Materials and Tools for Cultivating Fungus on Rice to Produce Aflatoxin and Acrotoxin

1. Sterile distilled water 500 ml
2. Triton × 100
3. Cotton
4. Sampler
5. 10-ml graduated sterile cylinder

To produce aflatoxin, first we mixed 10 ml of sterilized distilled water with 5µl of Triton ×100, then added it to the plate containing Aflatoxin Aspergillus Flavus NRRL 2999 productive fungus and scratched the surface of the fungus to completely mix the fungus with distilled water. After completing this, we added 500µl of the mixed fungus and distilled water to the autoclave rice that were prepared before. Then, we tightly shut the door of the Arlene and shook it until the solution containing fungus is completely spread over the rice. Next, incubated Arlene containing rice and fungus were kept at 28°C for 21 days. After 72 hours, when fungal myceliums grew on rice, we shook the Arlene and the rice to link them with each other due to the growth of the fungus break. This activity was performed after every 72 hours, 4 times a day. This will provide the needed space to grow fungus on rice. In order to produce acrotoxin from Aspergillus acrasus NRRL 3174, first 10 ml of sterilized distilled water was completely mixed with 5µl of Triton ×100, then the plate containing the aflatoxin Aspergillus acrasus NRRL 3174 productive fungus is added to it. Moreover, the surface of the fungus is scratched completely and the fungus is mixed with distilled water. After completing this, we added 500µl of mixed fungus and distilled water to the autoclaved rice that were already prepared. Then, the door of the Arlene is tightly closed and the solution is shook until the fungus completely spread over the rice. Then, the Arlene containing rice and fungus are incubated at 28°C for 21 days. After 72 hours, when fungal myceliums grow on rice, the Arlene and rice are shook to link each other due to the growth of the fungus break. The procedure is repeated after every 72 hours 4 times a day. This provided the needed space to grow fungus on rice. After the incubation period ended, we placed Arlene containing fungus for 20 minutes at 121°C and 15 psi pressure in the autoclave.

2.2.2. Toxin Maintaining Method

In order to maintain produced aflatoxin and to prevent molding, it should be poured into the metal trays separately after the incubation period and the produced aflatoxin and acrotoxin autoclaved and spread inside the tray completely. Then, we placed trays containing toxin in the aven at 80°C for 24 hours, after that we removed trays containing aflatoxin and acrotoxin from the oven. After that, contaminated rice with aflatoxin and acrotoxin were powdered separately by the mill and each one is inserted into the zipper plastic bags separately and left in the refrigerator (Fig. **1**).

(A) (B)

Fig. (1). Aflatoxin (**A**) and Ocrotoxin (**B**) produced by *Aspergillusflavus* NRRL2999 and *Aspergillusacrasus* NRRL 7431, receptively, in temperature of 28°C for 21 d incubation.

2.3. Mycotoxins Assay

The current study to determine AF and OTA concentrations used thin-layer chromatography (TLC) to separate non-volatile mixtures with high performance, the method described by Pons and Goldblatt in 1965 [12] by comparing the fluorescence with the standard. Diffusible zones of AFs and OTA were detected under long wave (365 nm) UV light as blue fluorescent.

2.4. Statistical Analysis

Each sample was analyzed in triplicate using the SPSS 17.0 software and data were calculated for comparison. The results were expressed as the mean ± standard deviation (SD).

3. RESULTS

The data obtained in the study are given in Table **1**. The mean levels of AF and OTA estimated in this investigation using PDA as a culture medium and TLC in rice samples were found to be 625 and 478.67 µg/kg of substrate used at 28°C in still culture, receptively. These concentrations were harmonic with the reports of the Iran National Standards Organization about the mycotoxin contamination of cultivated rice.

Table 1. Levels of aflatoxin and ochratoxin by *Aspergillus* strains isolated from rice.

Toxins	*Aspergillus* Strain	Concentration (µg/g)		Total
Aflatoxin	*Flavus*	B1	531	625
		B2	43	
		G1	21	
		G2	30	
Ochratoxin	*Acrasus*	Ochratoxin A	478.67	482.67
		Ochratoxin B	3	
		Ochratoxin C	1	

4. DISCUSSION

In the current study, the author's efforts to design an appropriate method to control the level of mycotoxins in rice consumed in the Persian market to avoid the associated risk of human disorders. Our results revealed that AF and OTA are the predominant mycotoxins detected in rice grains. Moreover, these results alarm us about the potential risks of A. flavus to public health and economic issue. Results close to the results of this study have also been obtained in various previous studies [2, 13, 14]; however, a certain amount of contamination seems unavoidable at present.

A temperature regime of 28°C was ideal for AFs and OTA production. It has been reported that similar results were obtained in some previous studies. Reddy *et al.* [15] reported that 25°C is the optimum temperature for the production of aflatoxin-B1 by A. flavus isolated from discolored rice grains in the Indian market. The occurrence of mycotoxin contamination is further stimulated by ongoing global warming as reflected in some findings [16]. Furthermore, the Aspergillus genus to boost growth and mycotoxin production requires lower humidity [17]. These results suggest that future studies on the production of mentioned toxins should be conducted at the optimum temperature and humidity

Due to the variety of structures of mycotoxins, it is impossible to use one standard technique for analysis and/or detection [7]. Screening of samples contaminated with mycotoxins by TLC gets yielded qualitative or semi quantitative results [17]. TLC provides a cheaper alternative to LC-based methods and has an important role, especially in developing countries, for surveillance purposes and control of regulatory limits [18].

PDA as a culture medium allows good growth of *Aspergillus* strains because these fungus species have exhibited the ability to thrive in standard PDA.

CONCLUSION

Using the methods of PDA media and TLC has shown great potential for mycotoxin analysis and is likely to be popular in the cereals Persian market. A. flavus NRRL 2999 and A. acrasus NRRL 7431 are two important species of AFs and OTA producing, which are considered a source of corruption in rice.

LIST OF ABBREVIATIONS

PDA	=	Potato Dextrose Agar
TLC	=	Thin-layer Chromatograpy
AFs	=	Aflatoxins
OTA	=	Ochratoxin A

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Pleadin J, Frece J, Markov K. Mycotoxins in food and feed. Adv Food Nutr Res 2019; 89: 297-345. [http://dx.doi.org/10.1016/bs.afnr.2019.02.007] [PMID: 31351529]

[2] Santos AR, Carreiró F, Freitas A, *et al.* Mycotoxins contamination in rice: Analytical methods, occurrence and detoxification strategies. Toxins 2022; 14(9): 647. [http://dx.doi.org/10.3390/toxins14090647] [PMID: 36136585]

[3] Eslamizad S, Yazdanpanah H, Hadian Z, *et al.* Exposure to multiple mycotoxins in domestic and imported rice commercially traded in Tehran and possible risk to public health. Toxicol Rep 2021; 8: 1856-64. [http://dx.doi.org/10.1016/j.toxrep.2021.11.008] [PMID: 34820291]

[4] Xu R, Kiarie EG, Yiannikouris A, Sun L, Karrow NA. Nutritional impact of mycotoxins in food animal production and strategies for mitigation. J Anim Sci Biotechnol 2022; 13(1): 69. [http://dx.doi.org/10.1186/s40104-022-00714-2] [PMID: 35672806]

[5] Kananub S, Jala P, Laopiem S, Boonsoongnern A, Sanguankiat A. Mycotoxin profiles of animal feeds in the central part of Thailand: 2015-2020. Vet World 2021; 14(3): 739-43. [http://dx.doi.org/10.14202/vetworld.2021.739-743] [PMID: 33935421]

[6] Liew WPP, Mohd-Redzwan S. Mycotoxin: Its impact on gut health and microbiota. Front Cell Infect Microbiol 2018; 8(60): 60. [http://dx.doi.org/10.3389/fcimb.2018.00060] [PMID: 29535978]

[7] Turner NW, Subrahmanyam S, Piletsky SA. Analytical methods for determination of mycotoxins: A review. Anal Chim Acta 2009; 632(2): 168-80. [http://dx.doi.org/10.1016/j.aca.2008.11.010] [PMID: 19110091]

[8] Zareshahrabadi Z, Bahmyari R, Nouraei H, *et al.* Detection of aflatoxin and ochratoxin a in spices by high-performance liquid chromatography. J Food Qual 2020; 2020: 1-8. [http://dx.doi.org/10.1155/2020/8858889]

[9] Mazaheri M. Determination of aflatoxins in imported rice to Iran. Food Chem Toxicol 2009; 47(8): 2064-6. [http://dx.doi.org/10.1016/j.fct.2009.05.027] [PMID: 19481133]

[10] Feizy J, Beheshti HR, Fahim NK, Janati SSF, Davari G. Survey of aflatoxins in rice from Iran using immunoaffinity column clean-up and HPLC with fluorescence detection. Food Addit Contam Part B Surveill 2010; 3(4): 263-7. [http://dx.doi.org/10.1080/19440049.2010.516456] [PMID: 24779626]

[11] Fatemeh Z, Alireza K, Hojjatollah S. Evaluation of the correlation between Aflatoxin B1 production and *Aspergillus* contamination in rice bran from northern Iran. Afr J Microbiol Res 2011; 5(11): 1306-10. [http://dx.doi.org/10.5897/AJMR11.132]

[12] Pons WA Jr, Goldblatt LA. The determination of aflatoxins in cottonseed products. J Am Oil Chem Soc 1965; 42(6): 471-5. [http://dx.doi.org/10.1007/BF02540087] [PMID: 14300202]

[13] Reddy CS, Reddy KRN, Raja Kumar N, *et al.* Exploration o aflatoxin contamination and its management in rice. J Mycol Plant Pathol 2004; 34(3): 816-20.

[14] Pereira VL, Fernandes JO, Cunha SC. Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. Trends Food Sci Technol 2014; 36(2): 96-136. [http://dx.doi.org/10.1016/j.tifs.2014.01.005]

[15] Reddy KRN, Reddy CS, Muralidharan K. Characterization of aflatoxin b1 produced by aspergillus flavus isolated from discoloured rice grains. J Mycol Plant Pathol 2005; 35(3): 470-4.

[16] Zhao Y, Wang Q, Huang J, *et al.* Mycotoxin contamination and presence of mycobiota in rice sold for human consumption in China. Food Control 2019; 98: 19-23. [http://dx.doi.org/10.1016/j.foodcont.2018.11.014]

[17] Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C. Mycotoxin analysis: An update. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2008; 25(2): 152-63. [http://dx.doi.org/10.1080/02652030701765723] [PMID: 18286405]

[18] Gilbert J, Anklam E. Validation of analytical methods for determining mycotoxins in foodstuffs. Trends Analyt Chem 2002; 21(6-7): 468-86. [http://dx.doi.org/10.1016/S0165-9936(02)00604-0]