

Investigating the ochratoxin production potential of *Aspergillus spp* in Indian Rice: A Comprehensive Study

ABSTRACT

Rice (*Oryza sativa*), world's second most important cereal crop and most commonly consumed grains in the world. Though more than 100,000 species of fungi are known to exist, majority of mycotoxigenic fungi belong to the species of *Aspergillus*, *Penicillium*, and *Fusarium*. Among *Aspergillus spp*, *Aspergillus niger* and *Aspergillus ochraceus* are known to produce Ochratoxin A (OTA), a notable mycotoxin having adverse effects due to ubiquitous presence, renal toxicity and lengthy persistence. Totally eighty-one samples including unpolished and polished rice samples were collected in various districts of Tamil Nadu to identify ochratoxigenic fungi particularly *Aspergillus spp* and the ability of fungus to produce OTA was quantified by RP HPLC-FLD analysis. Among the fungal genera, *Aspergillus spp* recorded the predominant fungus in rice. Molecular confirmation of ochratoxigenic fungi *A. niger* and *A. ochraceus* isolates was performed by 18s rDNA analysis. OTA producing ability by RP HPLC-FLD analysis revealed *A. ochraceus* of section *Circumdati* recorded the highest OTA production than *A. niger* isolates of section *Nigri*. Among the *A. ochraceus* isolates, potent isolate AO 9 documented the highest OTA production (196.84 ng/g) followed by AO 6 (104.74 ng/g). Similarly, in *A. niger* AN 1 showed highest production of 2.82 ng/g followed by AN 5 isolate (1.25 ng/g) respectively. The occurrence of *Aspergillus spp* in rice and the subsequent ochratoxin production raise concern about possible health risk to animal and human environment upon consumption. Hence there is need for improved storage practices and regular monitoring to prevent the prevalence of *Aspergillus* fungus and OTA contamination in rice supply chain.

Key words: Ochratoxigenic fungi, Aspergillus spp, RP HPLC-FLD, Rice.

1. INTRODUCTION

India and China are considered as the main producers of rice worldwide, while India's production was 196.25 million metric tons during 2022. As of 2023/2024, India and Thailand were the [main exporters](#) of rice, however India had the highest export volume of 16.5 million metric tons. Among the total [global consumption of milled rice](#) in 2023/24, India ranked second with 118 million metric tons of rice consumption. In India, leading rice-producing states are West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Tamil Nadu, Bihar, Chhattisgarh, and Odisha. West Bengal with the rice production of 15.75 million tons, stands as the largest rice producing Indian state, followed by Uttar Pradesh, Punjab and Tamil Nadu. The area under cultivation in Tamil Nadu was 2.04 million hectares with production of 7.98 million tons. (Statista, 2024).

Every year, nearly 12 million tonnes of rice are contaminated with mycotoxin, including ochratoxin, which results in a 25 % global economic loss of agricultural commodities of which rice is major concern. According to FAO, 15 % of the rice harvest is lost annually, as a result of improper storage conditions that encourage the growth of fungi and mycotoxin contamination (Santos et al., 2022). Fungal infection and toxin production have a significant impact on rice's

nutritional value and quality, causing discolouration of the grains and viability loss, which completely reduce the rice's market worth (Pandey et al., 2023).

Fungal invasion may occur during plant growth in a contaminated environment (pre-harvest contamination) as well as during transport and storage (post-harvest contamination) (Reddy et al., 2008). Although rice crop may become infected with ochratoxin producing fungus prior to harvest, OTA problem occurs during storage and do not arise prior to harvest (Petzinger & Weidenbach, 2002). Rice is typically grown under flood irrigation, which creates high moisture conditions that encourage the growth of fungi. Improper storage condition combined with meteorological events like flooding and intense rain during harvest increase the fungal attack. Sun-drying of rice, commonly practiced by majority of the farmers but this method is insufficient to lower the moisture level, leaving rice more vulnerable to fungal invasion (Majeed et al., 2018).

Van der Merwe et al. (1965) first identified ochratoxins (OTs) in *Aspergillus ochraceus* cultures (Shotwell et al., 1969). The ochratoxigenic fungi in rice attributed to the genus *Aspergillus*, particularly section *Circumdati* (*A. ochraceus*), *Aspergillus* section *Nigri*, and a few *Penicillium* species which are responsible for the majority of OTA production (Zhihong et al., 2015). The most critical factors which are essential for the growth of fungus and OTA production are water activity, temperature, moisture content and nutrients. For rice grain or seed stored for two to three weeks, the recommended moisture level is 14 – 18 %; for eight to twelve months, it is 12 – 13 %; and for more than a year, it is less than 9 %. *Aspergillus* and other storage fungus can often grow at a temperature of 25 – 35 °C and a relative humidity of 70 – 90 % (Brugman and Mario, 2024). The optimal temperature required for the *A. ochraceus* growth and OTA production was 25 – 30 °C, and optimum water activity (a_w) for growth was 0.96 - 0.98 a_w and OTA production at 0.98 a_w . For *A. niger*, the optimum temperature and water activity was 35 °C and 0.99 a_w for growth, OTA production respectively (Manna and Kim, 2017).

The fungal infection and consequent OTA production reported to have serious ill effects on human beings. Nephrotoxicity, neurotoxicity, immunotoxicity, and cause of the deadly disease known as Balkan Endemic Nephropathy (BEN) are among the toxicological consequences of OTA on humans (Ei Khoury and Atoui, 2010). The International Agency for Research on Cancer has categorised OTA as a human carcinogen in the class 2B family (Mutlu-Ingok et al., 2020). Despite the significance of rice as a staple food for Indians and the documented report of ochratoxins, there is little data on toxigenic fungal occurrence in rice and its toxin production ability. Hence present study was carried out to identify the ochratoxigenic fungi particularly *Aspergillus spp* and the possible production potential of OTA by these fungi in Indian rice.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Sterile disposable plates (Himedia, Mumbai) were utilised for culture maintenance and were autoclaved twice at 121 °C for 15 minutes each time before being disposed of. Ochratoxin A standard purchased from Sigma, USA (1 mg/ml) were stored in screw-cap amber vials (Agilent Technologies, USA). Glass wares, such as funnels, beakers, and amber vials, used for ochratoxin A extraction, were washed by soaking in 1% NaOCl for two hours and then soaking in 5% acetone for half an hour.

2.2 Sample collection

Totally 81 samples including unpolished (n = 36) and polished rice (n = 45) which are intended for human consumption were collected randomly from wholesale, local retail market and petty shops of different districts of Tamil Nadu, India during 2022 – 2023. The purpose of sampling is to isolate and identify the ochratoxigenic fungi (*Aspergillus spp*) and to assess the ochratoxin A

(OTA) production potential by *Aspergillus spp* using RP HPLC-FLD analysis. The samples were collected in sterilized polythene bags, labelled and stored at 4 °C.

2.3 Identification of *Aspergillus spp* and molecular confirmation

Colonies of *Aspergillus spp* were identified and pure culture was maintained on Czapek Yeast Agar medium (CYA). Based on colony morphology, species level of *Aspergilli* was determined as described by Pitt and Hocking, 2009. The morphologically identified *Aspergillus* isolates were subjected to molecular confirmation by characterizing the ITS (Internal Transcribed Spacer) region.

DNA extraction from mycelial mat of *Aspergillus niger* and *Aspergillus ochraceus* was done based on CTAB method as described by Allen *et al.* (2006). Using phenol-chloroform mixture, DNA was purified and precipitated with ethanol. The amplification of ITS region was performed using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') primers. The amplified PCR products were sequenced by using sanger dideoxy sequencing method at Biokart, Bangalore. The identification of isolates was determined by comparing the acquired DNA sequences with the National Centre for Biotechnology and Information's (NCBI) database.

2.4. Quantification of toxigenic potentiality of *Aspergillus niger* and *Aspergillus ochraceus* by HPLC-FLD

2.4.1. Inoculum preparation

Spore suspension of *A. niger* and *A. ochraceus* was prepared from ten days old cultures grown in CYA medium at 25 °C. Spores were collected by addition of 10 ml of sterile distilled water containing 0.05% Tween 80 by rubbing the surface with a glass rod. The concentration of the spore suspension was adjusted to 10⁶ conidia/ml using a hemocytometer and the spore suspension was used for further experiment (Abdel-Hadi and Magan, 2009).

2.4.2. Extraction of OTA

To determine the OTA producing ability of *Aspergillus niger* and *Aspergillus ochraceus* isolates, agar plug method was adapted as reported by Bragulat *et al.* (2001) and documented by using RP HPLC-FLD analysis. 10 µl of spore suspension was inoculated centrally onto the plates containing CYA medium and incubated for 15 days under dark at 25 °C. Five agar plugs were removed aseptically from the internal, middle and external areas of the well grown fungal colony along with mycelium using a cork borer (6 mm diameter). For OTA extraction, the agar plugs were transferred to sterile 2 ml eppendorf tube and one ml of HPLC grade methanol was added. The mixture was homogenized by vortexing for one min and followed by 60 mins incubation. The mixture was then centrifuged at 10,000 rpm for 5 mins. The supernatant was separated carefully and filtered using Millex® syringe filter (0.22 µm). The methanol extract was dried under nitrogen gas at 35 °C and re-dissolved in methanol: water (50:50). Fifty microlitre sample was injected into a reverse- phase High-performance liquid Chromatography (RP HPLC) instrument (Agilent 1200 HPLC system, Agilent Technologies, USA).

2.4.3. HPLC condition

Ochratoxin A was determined by reverse- phase HPLC (Agilent 1200 HPLC system, Agilent Technologies, USA) equipped with fluorescence detector and auto sampler. The silica packed C18 column (250mm × 4.6mm, 5µm particle size; Agilent Technologies, USA) used for chromatographic separation was maintained at 40 °C. The mobile phase was prepared with the

mixture of acetonitrile, water and acetic acid (51:47:2). The flow rate of mobile phase was maintained at 1 ml/ min. The excitation and emission wavelength of FLD detection were 333 and 460 nm respectively. Fifty microlitre from each sample was used as an injection volume.

2.5 Statistical analysis

All data presented are means of three replicates, and each experiment was repeated thrice. Differences between means were evaluated with analysis of variance using the Tukey means separation test at 5% significance. (Gomez and Gomez, 1984). The statistical analysis was performed using SPSS software version 20.0 for Windows (SPSS Inc.).

3. RESULTS AND DISCUSSION

The main focus of study was to enumerate the ochratoxigenic fungi (*Aspergillus spp*) in rice and its production potential of OTA. In the present findings, the species level of ochratoxigenic *Aspergillus* was identified based on molecular characterization. The molecular confirmation was done by 18S rDNA gene sequence analysis and the acquired sequence were submitted in NCBI database. Accordingly, ten isolates of *Aspergillus niger* and *Aspergillus ochraceus* were identified, subcultured on Czapek Yeast Agar medium (CYA) and incubated at 25 °C for 15 days under dark in order to analyse their OTA production.

The results of obtained accession number for ten isolates of *A. niger* and *A. ochraceus* were elucidated in Table 1, 2. As per the report of Gil Serna et al., 2009 18srDNA sequence analysis are widely used tool to differentiate the fungal species. The present investigation for the prevalence of ochratoxigenic fungi *A. niger* and *A. ochraceus* in rice samples are in accordance with the report of Pitt and Hocking, 1997 who reported the *Aspergillus* section Nigri and Circumdati being the major OTA producer in cereals such as rice.

The current findings are in line with previous studies of several authors who reported the occurrence of ochratoxigenic fungi *A. ochraceus* in rice. Accordingly, among 457 strains of *A. ochraceus* isolated from Japanese moldy rice, two strains were recorded as highly ochratoxin A producers. (Yamazaki et al., in 1970). Similarly, Varga et al., 2004 documented the presence of *A. ochraceus* in rice and van der Merwe et al., 1965 isolated *A. ochraceus* from African cereals. Frisvad et al., 2004 documented *A. ochraceus* as the potent OTA producer in rice. The occurrence of *Aspergillus ochraceus* and ochratoxin A production in rice was reported by Uchiyama et al. (1976).

From Figure 3 and 4 of present study shows that *A. ochraceus* isolates were capable of producing higher OTA production potential than *A. niger* isolates, as determined by RP HPLC-FLD analysis. The concentration of OTA produced by *A. ochraceus* isolates was varied in the range of 12.33 - 196.84 ng/g, while in *A. niger*, the production lies in the range of 0.25 - 2.82 ng/g respectively. *A. ochraceus* isolate AO 9 recorded the highest OTA production of 196.84 ng/g followed by AO 6 isolate (104.74 ng/g). The potent OTA producer in *A. niger* was AN 1 which recorded 2.82 ng/g followed by AN 5 isolate (1.25 ng/g) respectively. Similarly, Bragulat et al., 2001 reported the production of OTA by *A. niger* isolates in CYA media. The current result was in accordance with the findings of Mateo, 2018 who documented that *A. ochraceus* is the most potent ochratoxigenic species among *Aspergilli* and the second most OTA producing species belong to the section Nigri which includes *A. niger*.

The results and findings of several authors were supportive to our current investigation about the production of ochratoxin A by *A. ochraceus* and *A. niger* in rice samples. Accordingly, Zhihong et

al., 2015 reported *Aspergillus* section Nigri contamination of cereal grains including rice is a major issue in China. In connection with our study, Ochratoxigenic fungi *A. ochraceus* isolated from cereals reported to be potent OTA producer. (Mannaa and Kim, 2017). Laut et al., 2023 stated that polished rice was contaminated with 80 % of *Aspergillus* spp and reported prevalence of *A. niger*, *A. ochraceus* in rice. As per the report of Raghu et al., 2020, occurrence of *A. niger* in rice samples are documented in India. Han et al., 2019 studied the ochratoxin producing ability of 27 strains of *A. niger* for four weeks and results shown nine strains found to be OTA producer. Rice in tropical Asia is mostly contaminated with *Aspergillus* fungi such as *A. ochraceus* and OTA is considerably prevalent in rice. (Santos et al., 2022). The findings of Sayed et al., 2019 documented the occurrence of *Aspergillus ochraceus* (6.66 %) and *Aspergillus niger* (40 %), in rice samples.

Table 1. Molecular confirmation of *Aspergillus niger* isolates

S.No.	Isolate	Organism	Per cent identity	Accession number
1	AN 1	<i>Aspergillus niger</i>	99.99	PP972327
2	AN 2	<i>Aspergillus niger</i>	98.50	PQ041726
3	AN 3	<i>Aspergillus niger</i>	98.50	PQ041957
4	AN 4	<i>Aspergillus niger</i>	99.50	PQ041979
5	AN 5	<i>Aspergillus niger</i>	99.99	PQ043199
6	AN 6	<i>Aspergillus niger</i>	99.50	PQ044824
7	AN 7	<i>Aspergillus niger</i>	99.00	PQ044826
8	AN 8	<i>Aspergillus niger</i>	99.00	PQ046209
9	AN 9	<i>Aspergillus niger</i>	99.00	PQ047131
10	AN 10	<i>Aspergillus niger</i>	99.00	PQ047139

Table 2. Molecular confirmation of *Aspergillus ochraceus* isolates

S.No.	Isolate	Organism	Per cent identity	Accession number
1	AO 1	<i>Aspergillus ochraceus</i>	99.50	PQ097751

2	AO 2	<i>Aspergillus ochraceus</i>	98.50	PQ097749
3	AO 3	<i>Aspergillus ochraceus</i>	99.99	PQ097748
4	AO 4	<i>Aspergillus ochraceus</i>	98.00	PP973738
5	AO 5	<i>Aspergillus ochraceus</i>	99.99	PQ097715
6	AO 6	<i>Aspergillus ochraceus</i>	99.99	PQ097712
7	AO 7	<i>Aspergillus ochraceus</i>	99.00	PQ097294
8	AO 8	<i>Aspergillus ochraceus</i>	99.00	PQ041718
9	AO 9	<i>Aspergillus ochraceus</i>	99.99	PQ097668
10	AO 10	<i>Aspergillus ochraceus</i>	98.00	PP967837

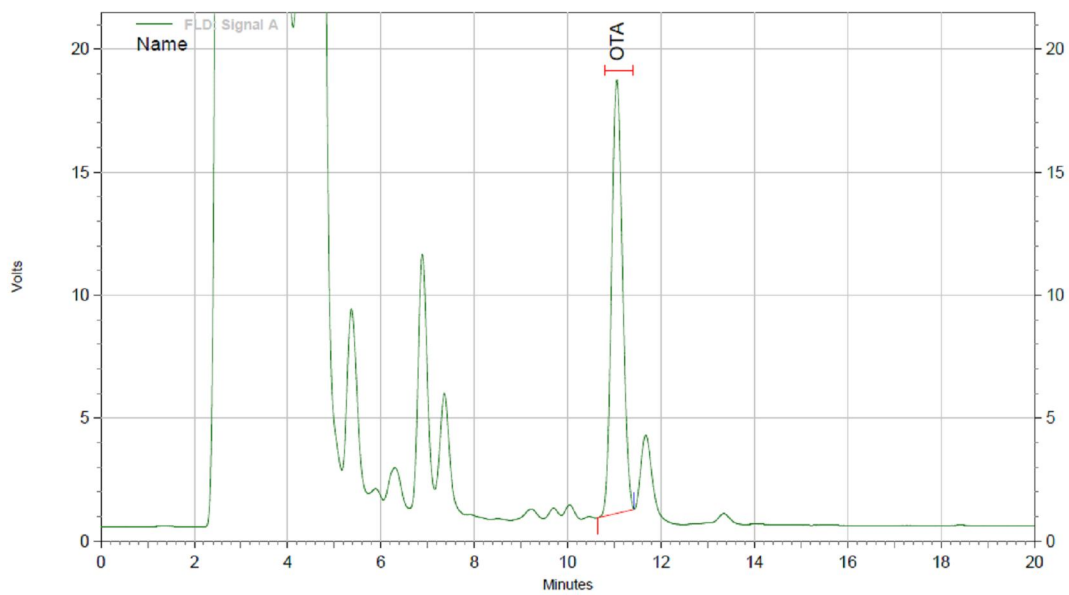


Fig 1: HPLC chromatogram of OTA production by *A. ochraceus* isolate A0 9

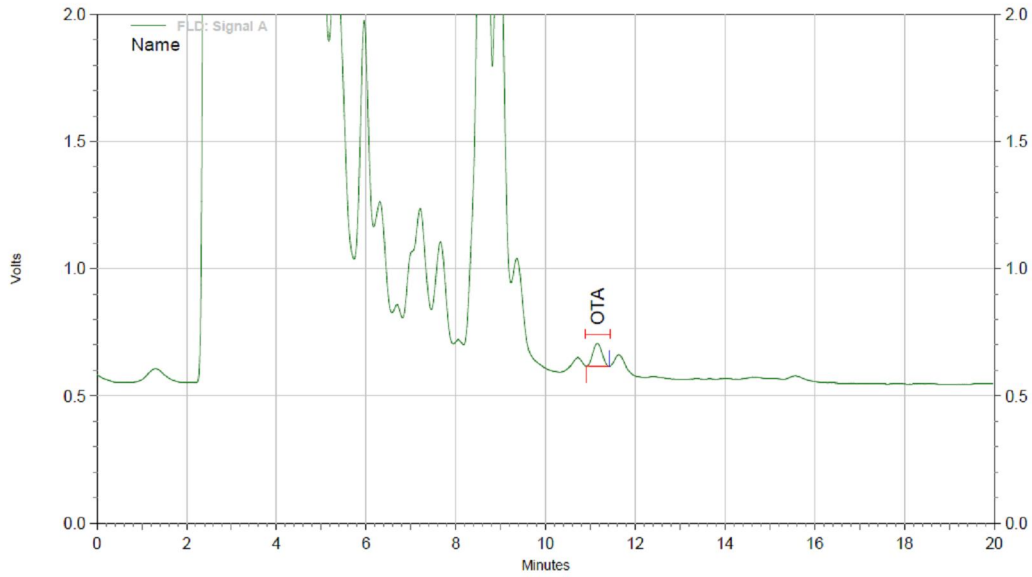


Fig 2: HPLC chromatogram of OTA production by *A. niger* isolate AN 3

Fig 3: Quantification of OTA production by *Aspergillus niger* isolates. Each value represents mean of three replicates.

Fig 4: Quantification of OTA production by *Aspergillus ochraceus* isolates. Each value represents mean of three replicates.

4. CONCLUSION

The present investigation documented the rice samples in Tamil Nadu, India was contaminated with ochratoxigenic fungi such as *A. ochraceus* and *A. niger*. Quantification by RP HPLC-FLD analysis revealed *A. ochraceus* being the highest OTA producer (196.84 ng/g) than *A. niger* which recorded 2.82 ng/g respectively. The prevalence of ochratoxigenic fungi and its toxin production ability suggest the possible production of ochratoxin A in rice samples which affect rice quality and nutritional value. Further, fungal infestation and toxin production deteriorate rice market value and pose serious ill effects to human health. These findings could be used as a reference, since fungal infection has a major effect on rice quality and food safety. Hence there is need for focus on improved storage practices and strict statutory limits have to be implemented to control the wide spread of fungal infection and ochratoxin production in rice.

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